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(54) Title: COMPOSITIONS AND METHODS TO TREAT GASTROINTESTINAL DISORDERS

(57) Abstract: The invention provides safe and effective methods for treating and preventing dysphagia, lower esophageal mucosal rings, esophageal strictures, achalasia, gastric mucosal injuries, and bacterial infections. The methods comprise administering at least one proton pump inhibitor, optionally in combination with antibacterial compounds. In one embodiment, the proton pump inhibitor is rabeprazole, a pharmaceutically acceptable salt thereof and/or a stereoisomer thereof.

Compositions and Methods To Treat Gastrointestinal Disorders

Related Applications

This application claims priority to U.S. Provisional Application No. 60/381,334 filed May 20, 2002, and to U.S. Provisional Application No. 60/331,585 filed November 16, 2001, the disclosures of which are incorporated by reference herein in there entirety.

Field of the Invention

The invention provides safe and effective methods for treating and preventing dysphagia, lower esophageal mucosal rings, esophageal strictures, achalasia, gastric mucosal injuries, and bacterial infections. The methods comprise administering at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a pharmaceutically acceptable salt thereof and/or a stereoisomer thereof.

Background of the Invention

Dysphagia is the inability to swallow or difficulty swallowing. Swallowing is a complicated action which is usually initiated voluntarily but always completed reflexively, whereby food is moved from the mouth through the pharynx and esophagus to the stomach. The act of swallowing occurs in three stages and requires the integrated action of the respiratory center and motor functions of multiple cranial nerves, and the coordination of the autonomic system within the esophagus. In the first stage, food is placed on the surface of the tongue. The tip of the tongue is placed against the hard palate. Elevation of the larynx and backward movement of the tongue forces the food through the isthmus of the fauces in the pharynx. In the second stage, the food passes through the pharynx. This involves constriction of the walls of the pharynx, backward bending of the epiglottis, and an upward and forward movement of the larynx and trachea. Food is kept from entering the nasal cavity by elevation of the soft palate and from entering the larynx by closure of the glottis and backward inclination of the epiglottis. During this stage, respiratory movements are inhibited by reflex. In the third stage, food moves down the esophagus and into the stomach. This movement is accomplished by momentum from the second stage, peristaltic contractions, and gravity. Although the main function of swallowing is the propulsion of food from the mouth into the stomach, swallowing also serves as a protective reflex for the upper

respiratory tract by removing particles trapped in the nasopharynx and oropharynx, returning materials refluxed from the stomach into the pharynx, or removing particles propelled from the upper respiratory tract into the pharynx. The absence of an adequate swallowing reflex greatly increases the chance of pulmonary aspiration.

In the past, patients suffering from dysphagia had to undergo dietary changes or thermal stimulation treatment to regain adequate swallowing reflexes. Thermal stimulation involves immersing a mirror or probe in ice or a cold substance. The tonsillar fossa is stimulated with the mirror or probe and the patient closes his mouth and attempts to swallow. While these traditional methods are usually effective for treating dysphagia, they often require that the patient endure weeks or months of therapy.

There is a need in the art for new and improved treatments for dysphagia and other esophageal disorders. The invention is directed to these, as well as other, important ends.

Summary of the Invention

The invention provides methods for treating and/or preventing dysphagia in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor. The methods can further comprise dilating the patient's esophagus; administering an endoscopic examination to the patient; and/or surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings or esophageal strictures.

The invention provides methods for treating and/or preventing lower esophageal mucosal rings or esophageal strictures in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor. The methods can further comprise dilating the patient's esophagus; administering an endoscopic examination to the patient; and/or surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings or esophageal strictures.

The invention provides methods for reducing or eliminating a patient's need for dilation of lower esophageal mucosal rings or esophageal strictures by administering a therapeutically effective amount of at least one proton pump inhibitor.

The invention provides methods for reducing or eliminating a patient's need for surgically incising, rupturing and/or excising the patient's lower esophageal mucosal

rings or esophageal strictures by administering a therapeutically effective amount of at least one proton pump inhibitor.

The invention provides methods for treating and/or preventing achalasia in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor. The methods can further comprise dilating the patient's esophagus; and/or administering an endoscopic examination to the patient.

The invention provides methods for treating and/or preventing gastric mucosal injuries in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor.

The invention provides methods for treating and/or preventing bacterial infections in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor and, optionally, at least one antibacterial compound.

The invention provides methods for modulating bacterial growth *in vitro* and/or *in vivo* by administering an effective amount of at least one proton pump inhibitor, and, optionally, at least one antibacterial compound.

The invention is described in more detail below.

Detailed Description of the Invention

Esophageal dilation is a technique used to stretch and/or fracture a blocked portion(s) of the esophagus. Several types of esophageal dilators are available including, for example, simple dilators (e.g., bougies, mercury-filled bougies, tapered-tipped Maloney dilators, blunt-tipped Hurst dilators), guided wire bougies (e.g., polyvinyl bougies, Savary dilators), balloon dilators (e.g., passed over a guidewire or through an endoscope), and achalasia dilators. Simple dilators are a series of flexible dilators of increasing thickness. One or more of the dilators can be passed down through the patient's esophagus at a setting. It is a simple method of stretching and/or fracturing the blockage in the patient's esophagus. In a guided wire bougie, the physician can perform an endoscopy and place a flexible wire across the stricture. The scope can be removed and the wire can be left in place. At least one dilator with a hole through it from end to end can be guided down the esophagus and across the stricture. At the end of the procedure, the wire can be removed. In balloon dilation, a deflated balloon can be placed through the endoscope and across the stricture in the esophagus. When the balloon is inflated, it stretches and/or fractures the stricture. An achalasia

dilator is similar to balloon dilation, but uses a larger balloon dilator, generally under x-ray control. In achalasia dilation, the spastic muscles in the lower esophagus are stretched and/or fractured with the balloon dilator.

A patient's esophagus can become blocked by, for example, esophageal strictures (e.g., peptic strictures), Schatzki's rings, ingestion of caustic agents, achalasia, tumors, or heredity. Esophageal strictures can be caused by acid reflux and/or a hiatal hernia which inflames and/or scars the esophagus. The fibrous scar contracts and narrows the esophageal opening. Schatzki's rings are narrow rings of benign fibrous tissue that constrict the lower esophagus. Schatzki's rings are also referred to as lower esophageal mucosal rings, and lower esophageal (Schatzki) rings. Achalasia is a persistent and marked spasm of the lower esophageal muscle which results in a persistent blockage of the esophagus.

"Patient" includes animals, preferably mammals, more preferably humans. "Patient" includes infants, children and adults, and includes males and females.

The invention provides methods for treating and/or preventing dysphagia in a patient in need thereof comprising administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

The invention provides methods for treating and/or preventing dysphagia in a patient in need thereof comprising dilating the patient's esophagus (e.g., lower esophagus), and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt). The patient's esophagus can be dilated by any dilation procedure in the art, such as those described herein. In one embodiment, the patient's esophagus is dilated with a simple bougie. The dysphagia can be of any origin. In one embodiment, the dysphagia can be caused by lower esophageal mucosal rings (i.e., Schatzki's rings). In another embodiment, the dysphagia can be caused by esophageal strictures (e.g., peptic strictures, such as those caused by reflux esophagitis). In another embodiment, the dysphagia can be caused by achalasia. The methods can further comprise administering at least one proton pump inhibitor prior to dilating the patient's

esophagus. The methods can further comprise administering an endoscopic examination to the patient prior to, during, and/or after dilating the patient's esophagus. The methods can further comprise surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings or esophageal strictures before and/or after dilating the patient's esophagus.

The invention provides methods for treating and/or preventing lower esophageal mucosal rings in a patient in need thereof comprising administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

The invention provides methods for treating and/or preventing lower esophageal mucosal rings in a patient in need thereof comprising dilating the patient's esophagus (e.g., lower esophagus), and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. The patient's esophagus can be dilated by any dilation procedure in the art, such as those described herein. In one embodiment, the patient's esophagus is dilated with a simple bougie. The methods can further comprise administering at least one proton pump inhibitor prior to dilating the patient's esophagus. The methods can further comprise administering an endoscopic examination to the patient prior to, during, and/or after dilating the patient's esophagus. The methods can further comprise surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings before and/or after dilating the patient's esophagus.

The invention provides methods for treating and/or preventing esophageal strictures in a patient in need thereof comprising administering a therapeutically effective amount of at least one proton pump inhibitor. The esophageal strictures can be peptic strictures, such as those caused by reflux esophagitis. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

The invention provides methods for treating and/or preventing esophageal strictures in a patient in need thereof comprising dilating the patient's esophagus, and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. The patient's esophagus can be dilated by any dilation procedure in the

art, such as those described herein. In one embodiment, the patient's esophagus is dilated with a simple bougie. The methods can further comprise administering at least one proton pump inhibitor prior to dilating the patient's esophagus. The methods can further comprise administering an endoscopic examination to the patient prior to, during, and/or after dilating the patient's esophagus. The methods can further comprise surgically incising, rupturing and/or excising the patient's esophageal strictures before and/or after dilating the patient's esophagus.

The invention provides methods for treating and/or preventing dysphagia in a patient in need thereof comprising surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings, and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt). The methods can further comprise administering at least one proton pump inhibitor prior to surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings. The methods can further comprise administering an endoscopic examination to the patient prior to, during, and/or after surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings. The methods can further comprise dilating the patient's esophagus (e.g., lower esophagus) prior to and/or after surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings.

The invention provides methods for treating and/or preventing dysphagia in a patient in need thereof comprising surgically incising, rupturing and/or excising the patient's esophageal strictures, and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt). The methods can further comprise administering at least one proton pump inhibitor prior to surgically incising, rupturing and/or excising the patient's esophageal strictures. The methods can further comprise administering an endoscopic examination to the patient prior to, during, and/or after surgically incising, rupturing and/or excising the patient's esophageal strictures. The methods can further comprise dilating the patient's esophagus prior to and/or after surgically incising, rupturing and/or excising the patient's esophageal strictures.

The invention provides methods for treating and/or preventing lower esophageal mucosal rings in a patient in need thereof comprising surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings, and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt). The methods can further comprise administering at least one proton pump inhibitor prior to surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings. The methods can further comprise administering an endoscopic examination to the patient prior to, during, and/or after surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings. The methods can further comprise dilating the patient's esophagus (e.g., lower esophagus) prior to and/or after surgically incising, rupturing and/or excising the patient's lower esophageal mucosal rings.

The invention provides methods for treating and/or preventing esophageal strictures in a patient in need thereof comprising surgically incising, rupturing and/or excising the patient's esophageal strictures, and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt). The methods can further comprise administering at least one proton pump inhibitor prior to surgically incising, rupturing and/or excising the patient's esophageal strictures. The methods can further comprise administering an endoscopic examination to the patient prior to, during, and/or after surgically incising, rupturing and/or excising the patient's esophageal strictures. The methods can further comprising dilating the patient's esophagus prior to and/or after surgically incising, rupturing and/or excising the patient's esophageal strictures.

The invention provides methods for reducing or eliminating a patient's need for dilation of lower esophageal mucosal rings or esophageal strictures comprising administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt). The methods can further comprise dilating the lower esophageal mucosal rings or esophageal strictures

before and/or after administering the therapeutically effective amount of at least one proton pump inhibitor. The methods can further comprise administering an endoscopic examination to the patient before, during, and/or after administering a therapeutically effective amount of at least one proton pump inhibitor.

The invention provides methods for reducing or eliminating a patient's need for surgically incising, rupturing and/or excising the lower esophageal mucosal rings or esophageal strictures comprising administering a therapeutically effective amount of at least one proton pump inhibitor. The methods can further comprising dilating and/or surgically incising, rupturing and/or excising the lower esophageal mucosal rings or esophageal strictures before and/or after administering the therapeutically effective amount of at least one proton pump inhibitor. The methods can further comprise administering an endoscopic examination to the patient before, during, and/or after administering a therapeutically effective amount of at least one proton pump inhibitor.

The term "eliminating" means that the methods of the invention prevent the need for any future dilation and/or surgical treatment due to lower esophageal mucosal rings or esophageal strictures. The term "reducing" means that the methods of the invention allow greater periods of time elapse between dilations and/or surgical treatments when compared to the amount of time that would elapse between dilations and/or surgical treatments without the methods of the invention.

The invention provides methods for treating and/or preventing achalasia in a patient in need thereof comprising administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

The invention provides methods for treating and/or preventing achalasia in a patient in need thereof comprising dilating the patient's esophagus, and subsequently administering a therapeutically effective amount of at least one proton pump inhibitor. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt). The patient's esophagus can be dilated by any dilation procedure in the art, such as those described herein. The methods can further comprise administering at least one proton pump inhibitor prior to dilating the patient's esophagus. The methods can further comprises

administering an endoscopic examination to the patient prior to, during, and/or after dilating the patient's esophagus.

In another embodiment, the invention provides methods for preventing and/or treating a gastric mucosal injury by administering a therapeutically effective amount of at least one proton pump inhibitor to a patient in need thereof. The gastric mucosal injury can be caused by, for example, alcohol (e.g., ethanol) and/or drugs (e.g., prescription drugs and/or street drugs). For example, the invention provides methods for treating ethanol-induced gastric mucosal injury by administering a therapeutically effective amount of at least one proton pump inhibitor to a patient in need thereof. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

In other embodiments, the invention provides compositions comprising at least one proton pump inhibitor and at least one antibacterial compound, and, optionally, at least one other proton pump inhibitor. The compositions comprise a pharmaceutically acceptable carrier. The invention provides pharmaceutical kits comprising at least one proton pump inhibitor and at least one antibacterial compound, and, optionally, at least one other proton pump inhibitor. In the kits of the invention, the at least one proton pump inhibitor, the at least one antibacterial compound, and, optionally, the at least one other proton pump inhibitor, are separate components in the kit or are in the form of a composition in the kit. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

In other embodiments, the invention provides methods for preventing and/or treating bacterial infections by administering to a patient in need thereof a therapeutically effective amount of at least one proton pump inhibitor. In another embodiment, the invention provides methods for preventing and/or treating bacterial infections by administering to a patient in need thereof a therapeutically effective amount of at least one proton pump inhibitor and at least one antibacterial compound. In other embodiments, the invention provides methods for preventing and/or treating bacterial infections by administering to a patient in need thereof a therapeutically effective amount of at least one proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof and/or a pharmaceutically acceptable salt thereof), at least one other proton

pump inhibitor, and, optionally, at least one antibacterial compound. The proton pump inhibitor, proton pump inhibitor and/or the antibacterial compound can be administered to the patient separately or in the form of a composition. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

In other embodiments, the invention provides methods for modulating bacterial growth *in vivo* and/or *in vitro* by administering an effective amount of at least one proton pump inhibitor. In another embodiment, the invention provides methods for modulating bacterial growth *in vivo* and/or *in vitro* by administering an effective amount of at least one proton pump inhibitor and at least one antibacterial compound. In other embodiments, the invention provides methods for modulating bacterial growth *in vivo* and/or *in vitro* by administering an effective amount of at least one proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof and/or a pharmaceutically acceptable salt thereof), at least one other proton pump inhibitor, and at least one antibacterial compound. The proton pump inhibitor, the antibacterial compound and/or the proton pump inhibitor can be administered separately or in the form of a composition. In one embodiment, the proton pump inhibitor is rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof (e.g., sodium salt).

"Modulating bacterial growth" includes inhibiting the growth of bacteria; reducing the rate at which the bacteria grows (i.e., compared to the rate at which untreated bacteria grows); and/or killing the bacteria. The growth of bacteria can be modulated *in vitro* and/or *in vivo*.

It has been unexpectedly discovered that the combined use of a proton pump inhibitor and at least one antibacterial compound enhances the effect of the antibacterial compound. The enhanced effect can be achieved by administering the compounds separately or in the form of a composition. The effect can be further enhanced by administering at least one other proton pump inhibitor.

When administered separately, the proton pump inhibitors and antibacterial compounds can be administered about the same time as part of an overall treatment regimen, i.e., as a combination therapy. "About the same time" includes administering the proton pump inhibitors and at least one antibacterial compound at the same time, at different times on the same day, or on different days, as long as they are administered

as part of an overall treatment regimen.

"Antibacterial compounds" include any antibacterial compounds and antibiotics in the art, including derivatives and metabolites thereof. Exemplary antibacterial compounds include beta-lactam compounds, quinolone compounds, cephalosporin compounds, carbapenem compounds, glycopeptide antibiotics, lipopeptide antibiotics, monobactam compounds, aminoglycoside antibiotics, streptogramin compounds, oxazolidinone compounds, macrolide compounds, azalide compounds, ketolide compounds, tetracycline compounds, lincosamide compounds, penicillin compounds, beta-lactamase inhibitors, efflux pump inhibitors, and the like. "Antibacterial compounds" include any other antibiotic, derivative thereof or metabolite thereof that does not fit into the categories described herein. The antibacterial compounds can be naturally produced and/or synthetically produced.

Any quinolone compound in the art can be used in the compositions and methods of the invention. "Quinolone compounds" includes fluoroquinolone compounds. Exemplary quinolone compounds include nalidixic acid, cinoxacin, oxolinic acid, norfloxacin, lomefloxacin, enoxacin, ofloxacin, ciprofloxacin, levofloxacin, sparfloxacin, gatifloxacin, grepafloxacin, gemifloxacin, sitafloxacin, moxifloxacin, trovafloxacin, alatrofloxacin, clinafloxacin, DC-756, Y-34867, T-3811, WQ-3034, S-34109, HSR-903, CFC-222, and the like.

Any cephalosporin compound in the art can be used in the compositions and methods of the invention. "Cephalosporin compounds" include cephem antibiotics and oxacephem antibiotics. Exemplary cephalosporin compounds include E1077, E1101, S-1090, FK041, MC-02,479, ME1209, Ro 63-9141, cefadroxil, cephalexin, cephradine, cephalothin, cephapirin, cefazolin, cefaclor, cefuroxime, cefprozil, loracarbef, cefamandole, cefoxitin, cefmetazole, cefotetan, cefonicid, cefixime, cefpodoxime, cefibuten, cefoperazone, cefotaxime, ceftizoxime, ceftazidime, cefdinir, ceftriaxone, cefepime, cefditoren pivoxil and the like. In one embodiment, the cephalosporin compound is E1077 or E1101.

Any carbapenem compound in the art can be used in the compositions and methods of the invention. Carbapenem compounds include penem compounds (e.g., MEN 10700). Exemplary carbapenem compounds include E1010, J-111,225, J-111,347, J-114,870, J-114,871, L-786,392, MK-826, S-4661, biapenem, sanfetrinem,

imipenem, meropenem, L-084, LJC 11,036, KR-21056, KR-21012, CS-834, R-95867, DZ-2640, DU-6681, GV 104326, MEN 10700, ritipenem, and the like. In one embodiment, the carbapenem compound is E1010.

Any glycopeptide and lipopeptide antibiotic in the art can be used in the compositions and methods of the invention. Exemplary glycopeptide and lipopeptide antibiotics include vancomycin, teicoplanin, BI 397, daptomycin, LY312607, LY314015, LY333328, and the like.

Any monobactam compound in the art can be used in the compositions and methods of the invention. Exemplary monobactam compounds include aztreonam.

Any aminoglycoside antibiotics in the art can be used in the compositions and methods of the invention. Exemplary aminoglycoside antibiotics include sisomicin, streptomycin, micronomicin, gentamicin, tobramycin, netilmicin, amikacin, kanamycin and the like.

Any streptogramin compound in the art can be used in the compositions and methods of the invention. Exemplary streptogramin compounds include virginiamycin and the like.

Any oxazolidinone compound in the art can be used in the compositions and methods of the invention. Exemplary oxazolidinone compounds include linezolid, PNU-107922, PNU-140457, PNU-172576, PNU-176798 and the like.

Any macrolide, azalide or ketolide compound in the art can be used in the compositions and methods of the invention. Exemplary macrolide, azalide and ketolide compounds include erythromycin, clarithromycin, troleandomycin, roxithromycin, dirithromycin, azithromycin, A-181785, A-184656, A-241550, CP-279,107, CP-544,372, HMR 3004-HMR 3647, TE-802, TE-810, telithromycin, erythromycin/sulfisoxazole and the like.

Any tetracycline compound in the art can be used in the compositions and methods of the invention. Glycylcycline compounds fall within the scope of tetracyclicine compounds. Exemplary tetracycline compounds include tetracycline, oxytetracycline, demeclocycline, methacycline, doxycycline, minocycline, terbutyl-minocycline, and the like.

Any lincosamide antibiotic in the art can be used in the compositions and methods of the invention. Exemplary lincosamide antibiotics include lincomycin,

clindamycin, pirlimycin and the like.

Any penicillin compound in the art can be used in the compositions and methods of the invention. Exemplary penicillin compounds include penicillin G, penicillin V, ampicillin, ampicillin/sulbactam, amoxicillin, amoxicillin/clavulanate, hetacillin, methicillin, cloxacillin, dicloxacillin, nafcillin, oxacillin, azlocillin, carbenicillin, mezlocillin, piperacillin, piperacillin/tazobactam, ticarcillin, ticarcillin/clavulanate, and the like.

Any beta-lactamase inhibitor in the art can be used in the compositions and methods of the invention. Exemplary beta-lactamase inhibitors include aztreonzm, cefotetan, loracarbef, cefoxitin, meropenem, imipenem/cilastatin, cefinase, clavulanate, sulbactam, tazobactam, and the like.

Any efflux pump inhibitor in the art can be used in the compositions and methods of the invention. Exemplary efflux pump inhibitors include CCCP, PSC-833, MC-04,124, MC-510027, MC-207110, MC-02595, and the like.

Any other antibiotic in the art not falling into one of the above categories can be used in the compositions and methods of the invention. Such other antibiotics can include trimethoprim-sulfamethoxazole, chloramphenicol, metronidazole, mupirocin, everninomicin, rifamycin, clindamycin, colistimethate, quinupristin/dalfopristin, vancomycin, and the like.

The invention also provides methods for potentiating the at least one proton pump inhibitor by further administering at least one beta-lactamase inhibitor. The at least one proton pump inhibitor and the at least one beta-lactamase inhibitor can by administered separately or in the form of a composition. The invention also provides methods for potentiating the combination of at least one proton pump inhibitor and at least one antibacterial compound (i.e., other than a beta-lactamase inhibitor) by further administering at least one beta-lactamase inhibitor. The at least one proton pump inhibitor, the at least one antibacterial compound, and the at least one beta-lactamase inhibitor can by administered separately or in the form of a composition. Any beta-lactamase inhibitor in the art can be used. Exemplary beta-lactamase inhibitors include aztreonzm, cefotetan, loracarbef, cefoxitin, meropenem, imipenem/cilastatin, cefinase, clavulanate, sulbactam, tazobactam, and the like. In one embodiment, the invention provides methods for administering a therapeutically effective amount of at least one

proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof), at least one antibacterial compound (i.e., other than a beta-lactamase inhibitor), and at least one beta-lactamase inhibitor to treat a bacterial infection in a patient in need thereof, or to modulate the growth of bacteria. The combinations can include, for example, rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof, amoxicillin and clavulanate; rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof, piperacillin and clavulanate; rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof, ticarcillin and tazobactam; or rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof, ampicillin and sulbactam. One skilled in the art will recognize that other combinations can be used.

The invention provides methods for potentiating the at least one proton pump inhibitor by further administering at least one efflux pump inhibitor. The at least one proton pump inhibitor and the at least one efflux pump inhibitor can be administered separately or in the form of a composition. The invention also provides methods for further potentiating the combination of at least one proton pump inhibitor and at least one antibacterial compound (i.e., other than an efflux pump inhibitor) by further administering at least one efflux pump inhibitor. The at least one proton pump inhibitor, the at least one antibacterial compound and the at least one efflux pump inhibitor can be administered separately or in the form of a composition. The efflux pump inhibitor can be any in the art. Exemplary efflux pump inhibitors include CCCP, PSC-833, MC-04,124, MC-510027, MC-207110, MC-02595, and the like. In one embodiment, the methods can comprise administering a therapeutically effective amount of at least one proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof), at least one antibacterial compound (e.g., a quinolone, such as levofloxicin), and at least one efflux pump inhibitor to treat a bacterial infection (e.g., *P. aeruginosa*) in a patient in need thereof, or to modulate bacterial growth (e.g., *P. aeruginosa*) *in vivo* or *in vitro*. In other embodiments, the methods can comprise administering a therapeutically effective amount of at least one proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof), at least one antibacterial compound (e.g., a macrolide, such as azithromycin, clarithromycin and/or erythromycin), and at least one

efflux pump inhibitor to treat a bacterial infection (e.g., *E. coli*, *H. influenzae*, *K. pneumoniae*) in a patient in need thereof, or to modulate bacterial growth (e.g., *E. coli*, *H. influenzae*, *K. pneumoniae*) *in vivo* or *in vitro*.

Bacterial infections that can be treated with the methods of the invention include any in the art including, for example, urinary tract infections, respiratory tract infections, skin infections, urethral infections, sexually transmitted diseases (e.g., gonococcal infections, chlamydial infections), bone infections, joint infections, infectious diarrhea, typhoid fever, prostatitis, sinusitis, chronic bronchitis, pneumonia, gastrointestinal infections (e.g., *H. pylori*), intra-abdominal infections, gynecologic and pelvic infections, anthrax, and the like.

Bacterial infections that can be treated in a patient can be infections caused by any bacteria in the art including, for example, *Helicobacter* species (e.g., *pylori*), *Clostridium* species (e.g., *difficile*, *botulinum*), *Mycobacterium* species, *Staphylococcus* species (e.g., *aureus*), *Pseudomonas* species (e.g., *aeruginosa*), *Streptoccus* species (e.g., *pneumoniae*, *pyogenes*), *Enterobacteriaceae* species, *Enterococcus* species, *Mycoplasma* species (e.g., *pneumoniae*), *Chlamydia* species (e.g., *pneumoniae*, *trachomatis*), *Bacteroides* species, *Bacillus* species (e.g., *anthracis*), *Enterobacter* species, *Klebsiella* species (e.g., *pneumoniae*), *Haemophilus* species (e.g., *influenzae*, *parainfluenzae*), *Moraxella* species (e.g., *catarrhalis*), *Proteus* species (e.g., *mirabilis*), *Acinetobacter* species, *Serratia* species, *E. coli*, and the like. In one embodiment, the methods are directed to preventing and/or treating infections caused by *Staphylococcus aureus*. In another embodiment, the methods are directed to preventing and/or treating infections caused by *Pseudomonas aeruginosa*. In other embodiment, the methods are directed to preventing and/or treating infections caused by *Helicobacter pylori*. In other embodiment, the methods are directed to preventing and/or treating infections caused by *Clostridium difficile*.

Bacteria that can be modulated *in vivo* and/or *in vitro* by the methods of the invention can be any in the art including, for example, *Helicobacter* species (e.g., *pylori*), *Clostridium* species (e.g., *difficile*, *botulinum*), *Mycobacterium* species, *Staphylococcus* species (e.g., *aureus*), *Pseudomonas* species (e.g., *aeruginosa*), *Streptoccus* species (e.g., *pneumoniae*, *pyogenes*), *Enterobacteriaceae* species, *Enterococcus* species, *Mycoplasma* species (e.g., *pneumoniae*), *Chlamydia* species

(e.g., *pneumoniae*, *trachomatis*), *Bacteroides* species, *Bacillus* species (e.g., *anthracis*), *Enterobacter* species, *Klebsiella* species (e.g., *pneumoniae*), *Haemophilus* species (e.g., *influenzae, parainfluenzae*), *Moraxella* species (e.g., *catarrhalis*), *Proteus* species (e.g., *mirabilis*), *Acinetobacter* species, *Serratia* species, *E. coli*, and the like. In one embodiment, the method is directed to modulating the growth of *Helicobacter pylori*. In one embodiment, the method is directed to modulating the growth of *Staphylococcus aureus*. In another embodiment, the method is directed to modulating the growth of *Pseudomonas aeruginosa*. In another embodiment, the method is directed to modulating the growth of *Clostridium difficile*.

The invention also provides methods for treating and/or preventing *H. pylori* in order to prevent and/or treat cancer by administering a therapeutically effective amount of at least one proton pump inhibitor and, optionally, at least one antibacterial compound. The proton pump inhibitor is preferably rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof. In other embodiments, at least one proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof) is administered in conjunction (i.e., separately or in the form of a composition) with at least one proton pump inhibitor other than rabeprazole, and, optionally, at least one antibacterial compound. The cancer can be any cancer in the art, but is generally a gastrointestinal cancer, such as gastric cancer, duodenal cancer, esophageal cancer, laryngeal cancer, and the like.

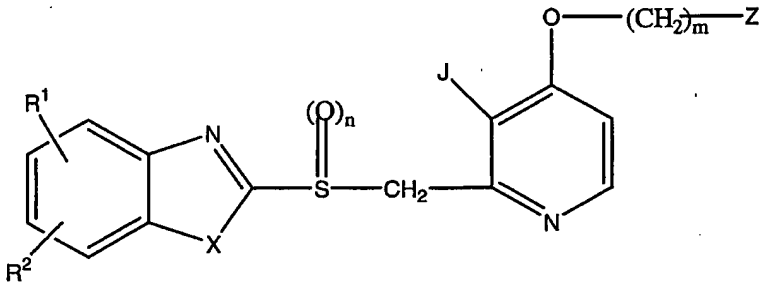
The invention provides methods for treating and/or preventing *H. pylori* in order to prevent and/or treat ulcers (e.g., esophageal ulcers, duodenal ulcers, stomach ulcers, jejenum ulcers, ileum ulcers) by administering a therapeutically effective amount of at least one proton pump inhibitor and, optionally, at least one antibacterial compound. The proton pump inhibitor is preferably rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof. In other embodiments, at least one proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof) is administered in conjunction (i.e., separately or in the form of a composition) with at least one proton pump inhibitor other than rabeprazole, and, optionally, at least one antibacterial compound.

The invention provides methods for treating and/or preventing *H. pylori* in order to prevent and/or treat dyspepsia by administering a therapeutically effective amount of

at least one proton pump inhibitor and, optionally, at least one antibacterial compound. The proton pump inhibitor is preferably rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof. In other embodiments, at least one proton pump inhibitor (e.g., rabeprazole, a stereoisomer thereof, and/or a pharmaceutically acceptable salt thereof) is administered in conjunction (i.e., separately or in the form of a composition) with at least one proton pump inhibitor other than rabeprazole, and, optionally, at least one antibacterial compound.

Any proton pump inhibitor in the art can be used in the compositions and methods described herein. Exemplary proton pump inhibitors include rabeprazole, omeprazole, lansoprazole, esomeprazole, pantoprazole and the like.

In one embodiment, the proton pump inhibitor is a pyridine derivative of formula (I), pharmaceutically acceptable salts thereof, and/or stereoisomers thereof:



(I)

wherein R^1 and R^2 are each independently a hydrogen atom, a halogen atom, a lower alkyl, lower alkoxy, halogenated lower alkyl, lower alkoxycarbonyl or carboxyl group;

X is -O-, -S- or =N-R^3, wherein R^3 is a hydrogen atom or a lower alkyl, phenyl, benzyl or lower alkoxycarbonyl group; and

Z is:

1. $-O(CH_2)_p-O-R^4$

   wherein p is an integer of 1 to 3 and R^4 is hydrogen atom or a lower alkyl, aryl or aralkyl group,

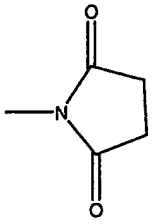
2. $-O-(CH_2)_q-R^5$

   wherein q is an integer of 1 to 3 and R^5 is a halogen atom or an alkoxycarbonyl, aryl or heteroaryl group,

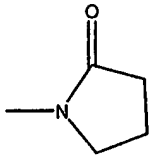
3. $-O-(CH_2)_r-O-(CH_2)_s-O-R^6$

wherein r and s are each independently an integer of 1 to 5 and R^6 is a hydrogen atom or a lower alkyl group,

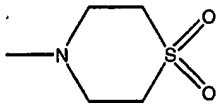
4.



5. 5.

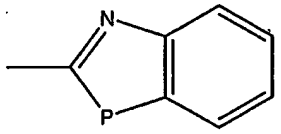


6.

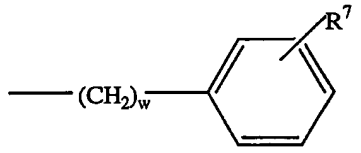


7. $-S(O)_t-A$

wherein t is an integer of 0 to 2, and A is a lower alkyl, alkoxycarbonylmethyl, pyridyl, furyl,



or



wherein B is -NH-, -O- or -S-, and w is an integer of 0 or 1;

8. $-N(R^8)-CH_2-C_6H_5$

wherein R^8 is an acetoxy or lower alkyl group;

9. $-OR^9$

wherein R^9 is a hydrogen atom, a lower alkyl or aryl group;

n is an integer of 0 to 2; m is an integer of 2 to 10, and J and K are each independently a hydrogen atom or a lower alkyl group, with the proviso that when Z is a group falling under the above category (9), then R^9 is a lower alkyl group and m stands for an integer of 3 to 10, and pharmaceutically acceptable salts thereof.

The same definitions for R^1, R^2, X, n, J, K, Z and m are used throughout the specification that follows and in the appended claim.

Also disclosed are pharmaceutical compositions containing one or more of these compounds as the active ingredient(s) in a pharmaceutically acceptable carrier, adjuvant or vehicle.

In the definition of the compounds of formula (I), the lower alkyl group defined with respect to R^1, R^2, R^3, R^4, R^6, R^7, R^8, A, J and K can be a straight-chain or branched alkyl group having 1 to 6 carbon atoms. Examples include methyl, ethyl, n-propyl, n-butyl, isopropyl, isobutyl, 1-methylpropyl, tert-butyl, n-pentyl, 1-ethylpropyl, isoamyl and n-hexyl groups, among which methyl and ethyl groups are most preferred.

The lower alkoxy group and the lower alkoxy moiety of the lower alkoxycarbonyl group defined above with respect to R^1 and R^2 can be an alkoxy group derived form the above lower alkyl group. Methoxy and ethoxy groups are most preferred.

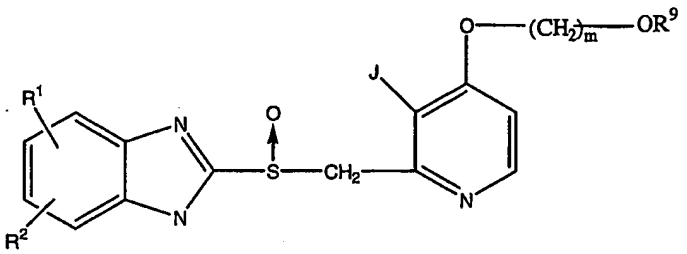
The halogen atom defined above includes chlorine, bromine, iodine or fluorine. The aryl group defined above with respect to R^4 and R^5 can be phenyl, tolyl, xylyl, napthyl or the like, which can be substituted with a lower alkoxy or hydroxyl group, a halogen atom or the like.

Examples of the arylalkyl defined above with respect to R^4 include benzyl and phenethyl groups.

Examples of the heteroaryl group defined above with respect to R^5 include pyridyl and furyl groups.

In the definition of Z in formula (I), groups 1, 2, 3, 4, 5 and 9 are preferred; and group 9 is the most preferred. As for R^1 and R^2, hydrogens for both and then a combination of a lower alkyl (e.g., methyl) for R^1 and hydrogen for R^2 are preferred. X is preferably $=NR^3$, where R^3 is hydrogen. A preferred value for n is 1. The preferred substituents for J and K are both hydrogen or where J is lower alkyl (e.g., methyl), and K is hydrogen, or when J is hydrogen and K is lower alkyl (e.g., methyl). Thus, J or K are independently preferably hydrogen or methyl, most preferably J is methyl and K is hydrogen.

In another embodiment, the compounds of formula (I) are compounds of formula (A), pharmaceutically acceptable salts thereof, and/or stereoisomers thereof:



(A)

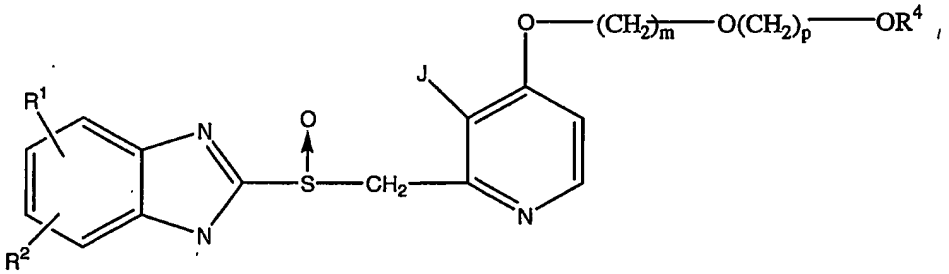
wherein R^1, R^2, J, m and R^9 have the same meanings as defined above.

In formula (A), the preferred R^1 and R^2 substituents are both hydrogen, or R^1 is 5-lower alkoxy, 5- lower alkyl or 5-halogenated lower alkyl and R^2 is hydrogen. The preferred substituent for J is hydrogen or methyl; the preferred value for m is in the range of 3 to 10, the most preferred being 3; and the preferred R^9 substituent is lower alkyl (e.g., methyl), or aryl. Among these possibilities for the compounds of formula (A), the preferred combination is when R^1 and R^2 are both hydrogen, J is methyl, m is 3 and R^9 is methyl.

Another group of preferred compounds in formula (A) are combinations of the above substituents where both R^1 and R^2 are hydrogen, J is hydrogen, m is 3 and R^9 is methyl.

Another group of preferred compounds falling within formula (A) is when both R^1 and R^2 are hydrogen, J is methyl, m is 2 and R^9 is benzyl.

In another embodiment, the compounds of formula (I) are compounds of formula (B), pharmaceutically acceptable salts thereof, and/or stereoisomers thereof:



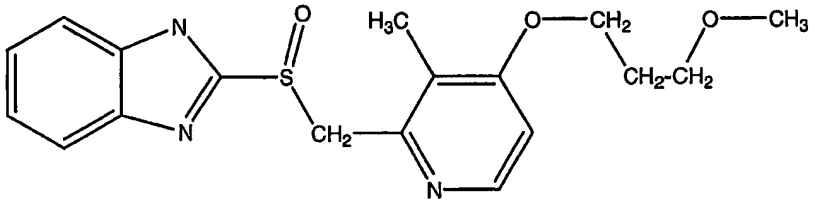
(B)

wherein R^1, R^2, J, p, m and R^4 have the same meanings as given above.

In formula (B), the preferred substituents for R^1 and R^2 are both hydrogen; or when R^1 is 5-lower alkoxy, 5-lower alkyl or 5- halogenated lower alkyl, R^2 is

hydrogen. The preferred value of m is 2 or 3; the preferred value for p is 2 or 3; and the preferred substituent for R^4 is methyl or benzyl. Of the above possibilities for formula (B), the most preferred combination is where R^1 is 5- methyl, R^2 is hydrogen, J is methyl, m is 2, p is 2 and R^4 is methyl.

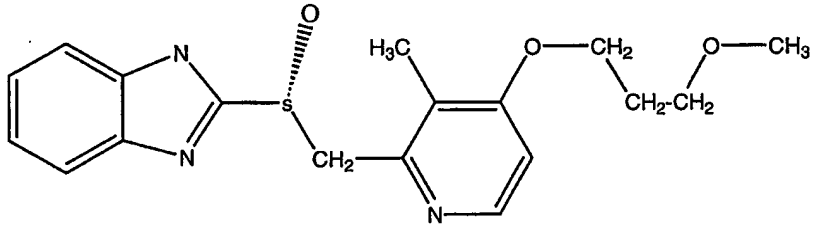
In another embodiment, the compound of formula I is a compound of formula (C), a pharmaceutically acceptable salt thereof, and/or a stereoisomer thereof:



(C).

Preferably, the compound of formula (C) is a sodium salt, which is known as rabeprazole sodium or ACIPHEX® (Eisai Inc., Teaneck, NJ).

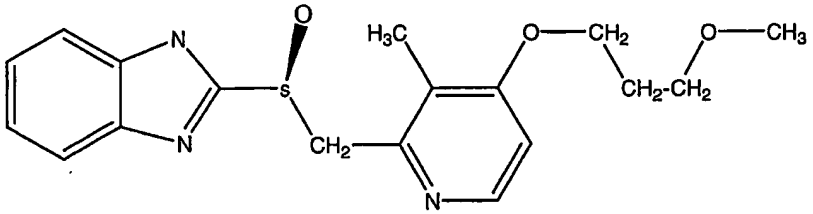
Although the compounds of the invention can be present as a hydrate or as a stereoisomer, the hydrates and stereoisomers are included within the scope of the invention. For example, the compound of formula (C) can be a compound of formula (D) or a pharmaceutically acceptable salt thereof (e.g., a sodium salt):



(D)

The compound of formula (D) is R (+) rabeprazole.

Alternatively, the compound of formula (C) can be a compound of formula (E) or a pharmaceutically acceptable salt thereof (e.g., a sodium salt):



(E)

The compound of formula (E) is S (-) rabeprazole.

The compounds of the invention can be administered as any pharmaceutically acceptable salt in the art. Pharmaceutically acceptable salts are known in the art and include those of inorganic acids, such as hydrochloride, sulfate, hydrobromide, sulfate, and phosphate; those of organic acids, such as formate, acetate, maleate, tartrate, trifluoroacetate, methanesulfonate, benzenesulfonate and toluenesulfonate, and those of amino acids such as arginine, aspartic acid and glutamic acid. When certain substituents are selected, the compounds of the invention can form, for example, alkali metal salts, such as sodium or potassium salts; alkaline earth metal salts, such as calcium or magnesium salts; organic amine salts, such as a salt with trimethylamine, triethylamine, pyridine, picoline, dicyclohexylamine or N,N'-dibenzylethylenediamine. One skilled in the art will recognize that the compounds of the invention can be made in the form of any of these or of any other pharmaceutically acceptable salt. For example, compounds represented by formula (I), wherein X is $=N-R^3$ and R^3 is a hydrogen atom, or compounds represented by formula (I), wherein Z is a group falling under the category 7 and B is a group of -NH-, can be present as a metal salt, such as sodium, potassium, magnesium or calcium.

The pyridine derivatives and proton pump inhibitors are commercially available and/or can be prepared by processes known in the art and described, for example, in U.S. Patent No. 5,045,552, the disclosure of which is incorporated by reference herein in its entirety. Rabeprazole sodium is commercially available as ACIPHEX® from Eisai Inc., Teaneck, NJ. Methods for preparing R (+) rabeprazole are described in WO 99/55157, the disclosure of which is incorporated by reference herein in its entirety. Methods for preparing S (-) rabeprazole are described in WO 99/55158, the disclosure of which is incorporated by reference herein in its entirety.

A therapeutically effective dosage regimen for treating the diseases described herein with the proton pump inhibitors and/or antibacterial compounds is selected in accordance with a variety of factors, including the age, weight, sex, and medical condition of the patient, the severity of the disease, the route of administration, pharmacological considerations such as the activity, efficacy, pharmacokinetic and toxicology profiles of the particular proton pump inhibitor and/or antibacterial compound, whether a drug delivery system is used and whether the proton pump

inhibitor and/or antibacterial compound is administered as part of a drug combination.

The proton pump inhibitors can be administered in amounts of about 0.01 to about 200 mg per day, preferably about 0.05 to about 50 mg per day, more preferably about 0.1 to about 40 mg per day, still more preferably about 10 to about 30 mg per day, most preferably about 20 mg per day. The compounds and/or compositions can be administered once a day or in divided doses, for example from 2 to 4 times a day, preferably once per day. One skilled in the art will recognize that when the compounds and/or compositions of the invention are administered to infants or children, the dose can be smaller than the dose administered to adults, and that the dose can be dependent upon the size and weight of the patient.

In preferred embodiments of the methods described herein, rabeprazole sodium, which is commercially available as ACIPHEX® (Eisai Inc., Teaneck, NJ), is administered as a delayed-release, enteric-coated tablet containing 20 milligrams rabeprazole sodium. The tablets can be administered one to about four times a day. In preferred embodiments, one 20 milligram ACIPHEX® tablet is administered once a day for the methods described herein. One skilled in the art will appreciate that when rabeprazole sodium is administered to infants or children, the dose can be smaller than the dose that is administered to adults.

The antibacterial compounds can be prepared by processes known in the art or can be obtained from commercial sources, and can be administered in therapeutically effective doses that are known in the art, such as those described in *The Physician's Desk Reference*.

The proton pump inhibitors and/or antibacterial compounds can be administered orally, topically, parenterally, by inhalation (nasal or oral), or rectally in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intrasternal injection, or infusion techniques. Preferably, the proton pump inhibitors are orally administered as tablets.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions can be formulated according to the known art using suitable dispersing or wetting agents, suspending agents (e.g., methylcellulose, Polysorbate 80, hydroxyethylcellulose, acacia, powdered tragacanth, sodium carboxymethylcellulose,

polyoxytehylene sorbitan monolaurate and the like), pH modifiers, buffers, solubilizing agents (e.g., polyoxyethylene hydrogenated castor oil, Polysorbate 80, nicotinamide, polyoxyethylene sorbitan monolaurate, Macrogol, an ethyl ester of castor oil fatty acid, and the like), preservatives and/or stabilizers. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be used are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally used as a solvent or suspending medium. For this purpose any bland fixed oil can be used including synthetic mono- or diglycerides, in addition, fatty acids such as oleic acid find use in the preparation of injectables. The preparations can be lyophilized by methods known in the art.

Solid dosage forms for oral administration can include capsules, tablets, sublingual tablets, powders, granules and gels; most preferably tablets. The solid dosage form can be a solid microencapsulated dosage, such as a microencapsulated powder, microencapsulated granules or a microencapsulated gel. A solid dosage form for oral administration can be prepared by mixing an active principle with filler and, if necessary, binder, disintegrating agent, lubricant, coloring agent, corrigent or the like and converting the obtained mixture into a tablet, coated tablet, granule, powder or capsule. Examples of the filler include lactose, corn starch, sucrose, glucose, sorbitol, crystalline cellulose and silicon dioxide, while those of the binder include polyvinyl alcohol, polyvinyl ether, ethylcellulose, methylcellulose, acacia, tragacanth, gelatin, shellac, hydroxypropylcellulose, hydroxypropylstarch and polyvinylpyrrolidone. Examples of the disintegrating agent include starch, agar, gelatin powder, crystalline cellulose, calcium carbonate, sodium hydrogencarbonate, calcium citrate, dextrin and pectin, while those of the lubricant include magnesium stearate, talc, polyethylene glycol, silica and hardened vegetable oils. The coloring agent can be any one which is permitted to be added to drugs. Examples of the corrigent include cacao powder, mentha herb, aromatic powder, mentha oil, borneol and powdered cinnamon bark. The tablets and granules can be, if necessary, coated with sugar, gelatin or the like. Preferably, the tablets have an enteric coating.

In other embodiments, the solid dosage form can be packaged as granules or a

powder in a pharmaceutically acceptable carrier, where the granules or powder are removed from the packaging and sprinkled on food or mixed with a liquid, such as water or juice. In this embodiment, the active compound can be mixed with flavoring or sweetening agents. The packaging material can be plastic, polyester films, nylon films, polyolefin films, shrink packing films, coated paper, or any material that prevents water or moisture from reaching the granules and/or powder.

Liquid dosage forms for oral administration can include pharmaceutically acceptable emulsions, solutions, suspensions, and syrups containing inert diluents commonly used in the art, such as water. The liquid dosage form can be a microencapsulated liquid, including microencapsulated emulsions, microencapsulated solutions, microencapsulated suspensions and microencapsulated syrups. Such compositions can also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

For administration by oral or nasal inhalation, the compounds and compositions can be delivered from an insufflator, a nebulizer or a pressured pack or other convenient mode of delivering an aerosol spray. Pressurized packs can include a suitable propellant. Alternatively, for administration by oral or nasal inhalation, the compounds and compositions can be administered in the form of a dry powder composition or in the form of a liquid spray.

Suppositories for rectal administration can be prepared by mixing one or more compounds or compositions with suitable nonirritating excipients, such as cocoa butter and/or polyethylene glycols, that are solid at room temperature and that melt at body temperature.

For topical administration to the epidermis, the proton pump inhibitors and/or antibacterial compounds can be formulated as ointments, creams or lotions, or as the active ingredient of a transdermal patch. The compounds and compositions can also be administered via iontophoresis. Ointments, creams and lotions can be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Alternatively, ointments, creams and lotions can be formulated with an aqueous or oily base and can also contain one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, and/or coloring agents. As creams or lotions, the proton pump inhibitors and/or antibacterial compounds can be mixed to

form a smooth, homogeneous cream or lotion with, for example, one or more of a preservative (e.g., benzyl alcohol 1% or 2% (wt/wt)), emulsifying wax, glycerin, isopropyl palmitate, lactic acid, purified water, sorbitol solution. Such topically administrable compositions can contain polyethylene glycol 400. To form ointments, the proton pump inhibitors can be mixed with one or more of a preservative (e.g., benzyl alcohol 2% (wt/wt)), petrolatum, emulsifying wax, and Tenox (II) (e.g., butylated hydroxyanisole, propyl gallate, citric acid, propylene glycol). Woven pads or rolls of bandaging material, e.g., gauze, can be impregnated with the transdermally administrable compositions for topical application.

The proton pump inhibitors and/or antibacterial compounds can also be topically applied using a transdermal system, such as one of an acrylic-based polymer adhesive with a resinous crosslinking agent impregnated with the proton pump inhibitors and laminated to an impermeable backing. For example, the proton pump inhibitors and/or antibacterial compounds can be administered in the form of a transdermal patch, such as a sustained-release transdermal patch. Transdermal patches can include any conventional form such as, for example, an adhesive matrix, a polymeric matrix, a reservoir patch, a matrix- or monolithic-type laminated structure, and are generally comprised of one or more backing layers, adhesives, penetration enhancers, and/or rate-controlling membranes. Transdermal patches generally have a release liner which is removed to expose the adhesive/active ingredient(s) prior to application. Transdermal patches are described in, for example, U.S. Patent Nos. 5,262,165, 5,948,433, 6,010,715 and 6,071,531, the disclosures of which are incorporated by reference herein in their entirety.

The invention provides pharmaceutical kits comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compounds and/or compositions of the invention, including, one or more proton pump inhibitors (e.g., rabeprazole, stereoisomers thereof and/or pharmaceutically acceptable salts thereof) and one or more antibacterial compounds. The proton pump inhibitors and/or antibacterial compounds can be separate components in the kit or can be in the form of a composition in the kit. The kits can also include, for example, other compounds and/or compositions, a device(s) for administering the compounds and/or compositions, and written instructions in a form prescribed by a governmental agency regulating the

manufacture, use or sale of pharmaceuticals.

While the proton pump inhibitors of the invention can be administered as the sole active pharmaceutical agent in the methods described herein, they can also be used in combination with one or more compounds which are known to be therapeutically effective against the specific disease that one is targeting for treatment.

Each of the patents and publications cited herein are incorporated by reference herein in their entirety.

It will be apparent to one skilled in the art that various modifications can be made to the invention without departing from the spirit or scope of the appended claims.

Claims

What is claimed is:

1. A method for treating and/or preventing dysphagia in a patient in need thereof by administering a therapeutically effective amount of at least one proton pump inhibitor.

2. The method of claim 1, further comprising, in any order, at least one of dilating the esophagus of the patient; administering an endoscopic examination to the patient; and surgically incising, rupturing and/or excising the lower esophageal mucosal rings or esophageal strictures of the patient.

3. A method for treating and/or preventing lower esophageal mucosal rings or esophageal strictures in a patient in need thereof by administering a therapeutically effective amount of at least one proton pump inhibitor.

4. The method of claim 3, further comprising, in any order, at least one of dilating the esophagus of the patient; administering an endoscopic examination to the patient; and surgically incising, rupturing and/or excising the lower esophageal mucosal rings or esophageal strictures of the patient.

5. A method for reducing or eliminating a patient's need for dilation of lower esophageal mucosal rings or esophageal strictures by administering to the patient a therapeutically effective amount of at least one proton pump inhibitor.

6. A method for reducing or eliminating a need for surgically incising, rupturing and/or excising lower esophageal mucosal rings or esophageal strictures in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor.

7. A method for treating or preventing achalasia in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor.

8. The method of claim 7, further comprising, in any order, at least one of dilating the esophagus of the patient; and administering an endoscopic examination to the patient.

9. A method for treating or preventing one or more gastric mucosal injuries in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor.

10. A method for treating or preventing a bacterial infection in a patient by administering a therapeutically effective amount of at least one proton pump inhibitor and, optionally, at least one antibacterial compound.

11. A method for treating or preventing *H. pylori* in a patient in need thereof to prevent or treat cancer in the patient by administering a therapeutically effective amount of at least one proton pump inhibitor and, optionally, at least one antibacterial compound.

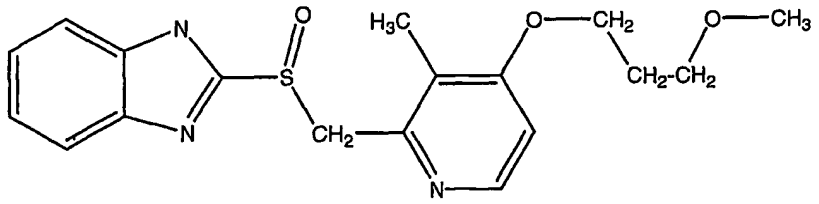
12. A method for treating or preventing *H. pylori* in a patient to prevent or treat an ulcer in the patient (e.g., esophageal ulcers, duodenal ulcers, stomach ulcers, jejenum ulcers, ileum ulcers) by administering a therapeutically effective amount of at least one proton pump inhibitor and, optionally, at least one antibacterial compound.

13. The method of claim 12, wherein the ulcer is an esophageal ulcer, a duodenal ulcer, a stomach ulcer, a jejenum ulcer, an ileum ulcer, or two or more thereof.

14. A method for treating or preventing *H. pylori* in a patient to prevent or treat dyspepsia in the patient by administering a therapeutically effective amount of at least one proton pump inhibitor and, optionally, at least one antibacterial compound.

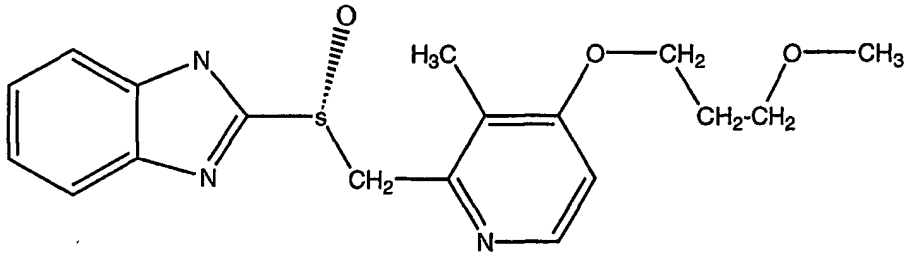
15. The method of claim 1, 3, 5, 6, 7, 9, 10, 11, 12 or 14, wherein the proton pump inhibitor is rabeprazole, omeprazole, esomeprazole, lansoprazole, pantoprazole, a pharmaceutically acceptable salt thereof and/or a steroisomer thereof.

16. The method of claim 1, 3, 5, 6, 7, 9, 10, 11, 12 or 14, wherein the proton pump inhibitor is a compound of formula (C) or a pharmaceutically acceptable salt thereof:



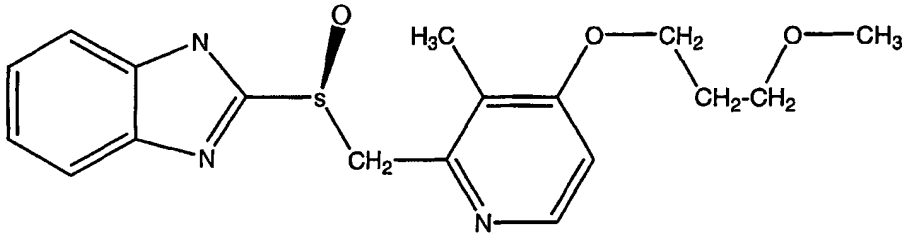
(C).

17. The method of claim 1, 3, 5, 6, 7, 9, 10, 11, 12 or 14, wherein the proton pump inhibitor is a compound of formula (D) or a pharmaceutically acceptable salt thereof:



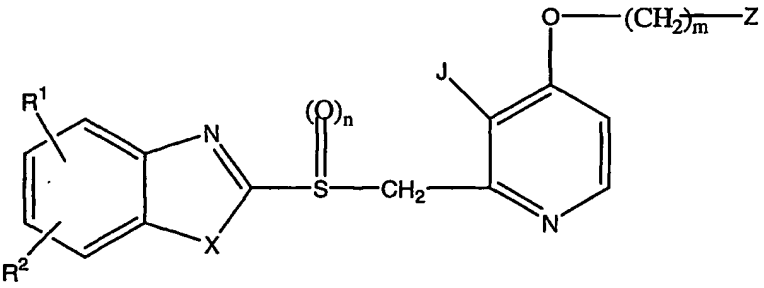
(D).

18. The method of claim 1, 3, 5, 6, 7, 9, 10, 11, 12 or 14, wherein the proton pump inhibitor is a compound of formula (E) or a pharmaceutically acceptable salt thereof:



(E).

19. The method of claim 1, 3, 5, 6, 7, 9, 10, 11, 12 or 14, wherein the proton pump inhibitor is a compound of formula (I), a pharmaceutically acceptable salt thereof, and/or a stereoisomer thereof:



(I)

wherein R^1 and R^2 are each independently a hydrogen atom, a halogen atom, a lower alkyl, lower alkoxy, halogenated lower alkyl, lower alkoxycarbonyl or carboxyl group;

X is -O-, -S- or =N-R^3, wherein R^3 is a hydrogen atom or a lower alkyl, phenyl, benzyl or lower alkoxycarbonyl group; and

Z is:

1. -$O(CH_2)_p$-O-R^4

wherein p is an integer of 1 to 3 and R^4 is hydrogen atom or a lower alkyl, aryl or aralkyl group,

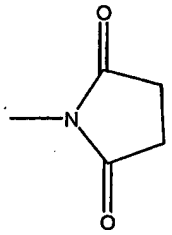
2. -O-$(CH_2)_q$-R^5

wherein q is an integer of 1 to 3 and R^5 is a halogen atom or an alkoxycarbonyl, aryl or heteroaryl group,

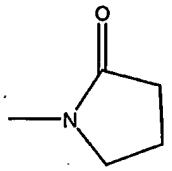
3. -O-$(CH_2)_r$-O-$(CH_2)_s$-O-R^6

wherein r and s are each independently an integer of 1 to 5 and R^6 is a hydrogen atom or a lower alkyl group,

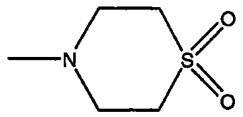
4.



5.

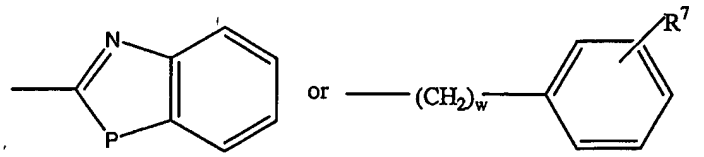


6.



7. -$S(O)_t$-A

wherein t is an integer of 0 to 2, and A is a lower alkyl, alkoxycarbonylmethyl, pyridyl, furyl,



wherein B is -NH-, -O- or -S-, and w is an integer of 0 or 1;

8. $-N(R^8)-CH_2-C_6H_5$

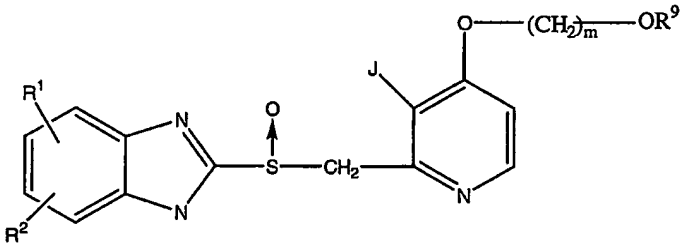
wherein R^8 is an acetoxy or lower alkyl group;

9. $-OR^9$

wherein R^9 is a hydrogen atom, a lower alkyl or aryl group;

n is an integer of 0 to 2; m is an integer of 2 to 10, and J and K are each independently a hydrogen atom or a lower alkyl group, with the proviso that when Z is a group falling under the above category (9), then R^9 is a lower alkyl group and m stands for an integer of 3 to 10.

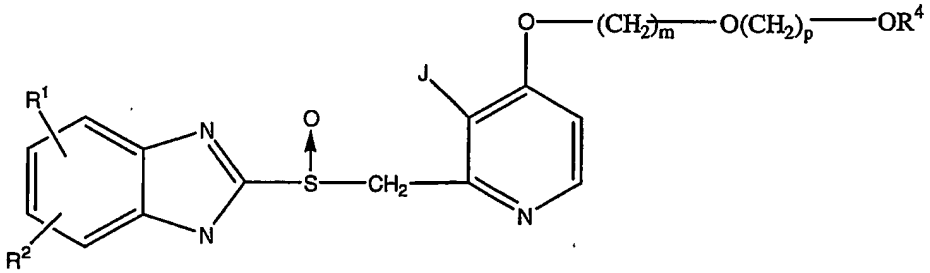
20. The method of claim 19, wherein the compound of formula (I) is a compound of formula (A):



(A)

wherein R^1, R^2, J, m and R^9 have the same meanings as defined above.

21. The method of claim 19, wherein the compound of formula (I) is a compound of formula (B):



(B)

wherein R^1, R^2, J, p, m and R^4 have the same meanings as given above.

22. The method of claim 10, 11, 12 or 14, wherein the antibacterial compound is selected from the group consisting of a beta-lactam compound, a quinolone compound, a cephalosporin compound, a carbapenem compound, a glycopeptide antibiotic, a lipopeptide antibiotic, a monobactam compound, an aminoglycoside antibiotic, a streptogramin compound, an oxazolidinone compound, a macrolide compound, an azalide compound, a ketolide compound, a tetracycline compound, a lincosamide compound, a penicillin compound, a beta-lactamase inhibitor, an efflux pump inhibitor, and a mixture of two or more thereof.

23. The method of claim 10, 11, 12 or 14, wherein the antibacterial compound is erythromycin or clarithromycin.

24. A therapeutic pharmaceutical combination comprising a therapeutically effective amount of rabeprazole, a pharmaceutically acceptable salt thereof and/or a stereoisomer thereof and a therapeutically effective amount of clarithromyin or erythromycin.

25. A therapeutic pharmaceutical combination comprising a therapeutically effective amount of rabeprazole, a pharmaceutically acceptable salt thereof and/or a stereoisomer thereof and a therapeutically effective amount of at least one antibacterial compound.

26. The therapeutic pharmaceutical combination of claim 25, wherein the antibacterial compound is selected from the group consisting of a beta-lactam compound, a quinolone compound, a cephalosporin compound, a carbapenem compound, a glycopeptide antibiotic, a lipopeptide antibiotic, a monobactam compound, an aminoglycoside antibiotic, a streptogramin compound, an oxazolidinone compound, a macrolide compound, an azalide compound, a ketolide compound, a tetracycline compound, a lincosamide compound, a penicillin compound, a beta-lactamase inhibitor, an efflux pump inhibitor, and a mixture of two or more thereof.

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(54) Title: MATRIX WITH IMMUNOMODULATING ACTIVITY

(57) Abstract

The invention claims an iscom matrix which is not a lipid vesicle comprising at least one lipid and at least one saponim but no intentional antigenic determinants and optionally also adjuvants for use as an immunomodulating agent, medicines, vaccines, kits containing the matrix and new saponins, and a process for preparing the new saponins. The invention also concerns a process for preparing the matrix characterized in that at least one sterol is solubilized in a solvent or detergent, the saponin or saponins are added, the other adjuvants and lipids are optionally also added, whereafter the organic solvent or the detergent may be removed for example with dialysis, ultra filtration, gel filtration or electrophoresis. The sterol and saponin might also be solubilized in the lipids and/or adjuvants.

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Matrix with immunomodulating activity

The present invention concerns an iscom matrix comprising at least one lipid and at least one saponin with immunomodulating effect, a process for preparing the matrix, a vaccine and a kit comprising the same and new saponins for incorporation in the matrix and a process for preparing the new saponins.

Many microbal and viral antigens can be produced by modern techniques today. Their full promise in vaccines will however not be realized unless they are administered along with an effective adjuvant, an agent that increases antibody and/or cell-mediated immune responses.

The only adjuvants currently authorized for human use in most countries are aluminium hydroxide and aluminum phosphate which have been used for many years to increase antibody responses to e.g. diphtheria and tetanus toxoids. Although these adjuvants are sufficient for many vaccines, studies have shown that other adjuvants, e.g. Freund's complete adjuvant (FCA), and Quil A often are more efficacious in eliciting antibody response and cell-mediated immunity in experimental animals. In fact,they are frequently required for protection. However, FCA produces granulomas at injection sites, which makes them unacceptable for human and veterinary vaccines. In fact, even aluminiumhydroxide may give rise to reactions in form of granuloma at the injection site. For these reasons, many attempts are made to develop adjuvants with the efficacy of FCA but without undesirable side effects.

In Morein's EPC Patent Applications Nos. 83850273.0 and 85850326.1, there are described immunogenic complexes between antigenic determinants with hydrophobic regions and glycosides, among them triterpensaponins and especially Quil A, so called iscom complexes. In such an iscom, the amount of Quil A

can be about 10-100 times lower and produce the same antigenic effect as when Quil A in free form is mixed with the antigen.

European Patent Application 87200035.1 indicates that the presence of antigen is not necessary for formation of the basic iscom structure, this being possible to form from a sterol, such as cholesterol, a phospholipid, such as phosphatidylethanolamine, and a glycoside such as Quil A.

It has now been discovered that a phospholipid is not needed for the preparation of the basic iscom structure including no antigen. Instead a sterol, such as cholesterol in conjunction with a glycoside such as Quil A are the essential structural components assembled into a complex resembling the typical cage-like iscom structure, so called matrix. It has also turned out that the matrix has immunomodulating effects such as adjuvant or immunosuppressive effect.

The present invention concerns a complex between at least one lipid such as a sterol, preferably cholesterol, and one or more saponins, such as triterpensaponins, especially Quil A or subcomponents thereof which is not a lipid vesicle without any intentional antigens or antigenic determinants for use as an immunomodulating agent. Thus, there is not integrated any antigenic component as is done in an iscom. This matrix has adjuvant effect and can be used mixed together with one or more antigens preferably in multimeric form.

In this iscom matrix there is also possible to integrate other adjuvants with hydrophobic regions. Addition of other lipids may be required to facilitate the inclusion of other adjuvants. Thus the present invention also concerns a complex containing lipids and adjuvants, other than cholesterol and saponins. Such complexes contains the matrix consisting of cholesterol and saponin, preferably Quil A or subcomponents thereof, one or more other adjuvants and one or more lipids

other than cholesterol. These are preferably not lipid vesicles or liposomes and have a very special structure in electron microscopy.

Liposomes have been described in the literature and their general structure is well known to biological research workers. Liposomes are vesicles comprising one or more series of lipid layers forming onion-like structures spaced one from another by aqueous material.

The matrix can be injected in an animal or human being as a mixture with the antigen in multimeric form. Alternatively the matrix and the antigen can be injected separately. In this case the best results are obtained if the adjuvant matrix and the the antigen are injected in regions which are drained into the same lymphatic gland. When the adjuvant is presentated in multimeric form in a matrix according to the invention the dose of adjuvant may be lowered as compared with when the adjuvant is injected separately in monomeric form or in an undefined form. This implies that toxical side effects caused by adjuvants when used conventionally, e.i. when they are injected alone as such, can be lowered or avoided. The dose of adjuvant can, however, not be lowered as much as is done in the iscom complexes according to the above mentioned patent applications.

When an adjuvant is used in a matrix according to the invention, the antigen is not integrated in the same particle as the adjuvant as is done in an iscom particle according to the above mentioned EPC Patent Applications. This implies that one can use antigens without amphiphatic properties or antigens which can not be forced to expose hydrophobic regions. As an example it can be mentioned that some viruses do not have amphiphatic proteins, e.g. picornavirus,adenovirus or parvovirus, but they have a form of submicroscopic particle with the antigen presentated i several copies, i.e. as multimers.

For such viruses it is more practical to inject them together with the new adjuvant complex than to couple hydrophobic groups to them or create hydrophobic groups by other means (e.g. partial denaturation) and integrate them into an iscom particle.

Typically, the present matrix contains sterol, preferably cholesterol, and one or more saponins in a molar ratio of about 1 to 1 or in a weight ratio of about 1 to 5. The complexes have an open sperical structure consisting of circular subunits or parts of the spherical structure revealed by electron microscopy. They have a sedimentation coefficient of about 20 S.

When other adjuvants are integrated, the lipid-adjuvant-matrix typically contains sterol and saponin in a molar ratio of about 1:1 and the other adjuvants and lipids together make up to about 1 molar. For such a matrix the molar ratio of sterol; saponin; other adjuvant and lipids is about 1:1:1. Thus the molar ratio of sterol; saponin; other adjuvant and other lipids is 1:1:0,1-1; 0,1-1, i.e. additional lipid or adjuvant may be present in the matrix until its molar ratio (or the sum of their molar ratios) is a half that of the saponin and sterol present.

The structure as revealed by electron microscopy is the same as for iscom and matrix (see Figure 1).

The sedimentation coefficient, being dependent on the density of material incorporated into the matrix, is about 12-22 S for matrixes containing cholesterol, saponin, other adjuvants and lipids.

The saponins can be any saponin with hydrophobic regions such as those described in R Tschesche and Wulf, Chemie und Biologie der Saponine in Fortschritte der Chemie Organischer

Naturstoffe, published by W Herz, H, Grisebach, G W Kirby, Vol 30 (1973), especially the strongly polar saponins, primarily the polar triterpensaponins such as the polar acidic bisdesmosides, e.g. saponin extract from Quillajabark Aralo-
5 side A, Chikosetsusaponin IV, Calendula-Glycoside C, Chiko-setsusaponin V, Achyranthes-Saponin B, Calendula-Glycoside A, Araloside B, Araloside C, Putranjia-Saponin III, Bersama-saponiside, Putrajia-Saponin IV, Trichoside A, Trichoside B, Saponaside A, Trichoside C, Gypsoside, Nutanoside, Dianthoside
10 C, Saponaside D, preferably aescine from Aesculus hippocasta-num (T Patt and W Winkler: Das therapeutisch wirksame Prinzip der Rosskastanie (Aesculus hippocastanum), Arzneimittelfor-schung 10(4), 273-275 (1960) or sapoalbin from Gyposophilla struthium (R Vochten, P Joos and R Ruyssen: Physico-chemical
15 properties of sapoalbin and their relation to the foam stabil-ity, J Pharm Belg 42, 213-226 (1968), especially saponin extract from Quillaja saponaria Molina, primarily the DQ--extract which is produced according to K Dalsgaard: Saponin Adjuvants, Bull Off Int Epiz 77 (7-8), 1289-1295 (1972) and
20 Quil A which is produced according to K Dalsgaard: Saponin Adjuvants III, Archiv für die Gesamte Virusforschung 44, 243-254 (1974). Quil A and subfragments thereof are preferred, especially the fragments B2, B3 and B4B described below.

25 The present invention also provides new glycosylated tri-terpenoid saponins derived from Quillaja Saponaria Molina of Beta Amyrin type with 8-11 carbohydrate moieties which have the following characteristics:

30 a) Substance B2 has a molecular weight of 1988, a carbon 13 nuclear magnetic resonance (NMR) spectrum as indicated in Figures 5A and 6A and a proton NMR spectrum as shown in Figures 11A and 12A.

35 b) Substance B3 has a molecular weight of 2150 and has a carbon 13 NMR spectrum as shown in Figures 5B and 6B, and a proton NMR spectrum as shown in Figures 11B and 12B.

c) Substance B4B has a molecular weight of 1862, a carbon 13 NMR spectrum as shown in Figures 5C and 6C, and a proton NMR structure as shown in Figures 11C and 12C.

Compounds B2 and B3 have adjuvant activity in their own right. The present invention also relates therefore to the use of these compounds as adjuvants. Compound B4B is of use in the preparation of an iscom matrix. B2 and B3 having adjuvant activity can be included in the matrix.

Matrix can be produced from a sterol such as cholesterol and the saponin B4B. Such a matrix does not seem to have any potent adjuvant activity. In order to potentiate the adjuvant activity in this matrix, it is possible and even preferable to integrate the saponins B2 and/or B3 and/or any other substance with adjuvant effect and with hydrophobic groups. If the adjuvants do not contain any hydrophobic groups such groups might be coupled to them by use of known chemical methods. If other adjuvants than B2 or B3 are to be integrated, there are preferably incorporated further lipids as listed on page 13, last paragraph and paragrahs 1-3 on page 14.

In the sterol-B4B matrix, it is also possible to integrate immunosuppressive substances containing hydrophobic groups or to which such groups have been coupled.

It is also possible to use the sterol-B4B matrix as an immunomodulating agent in mixture with adjuvants, immunosuppressive substances or antigens or mixtures thereof.

As immunodulating agents are considered substances that enhance, suppress or change the immune system such as adjuvants, suppressors, interleukins, interferons or other cytokins.

The invention preferably concerns an matrix containing a sterol, especially cholesterol, B4B and either of B2 and B3 or

both. When matrix is prepared from cholesterol and Quil A, it comprises B2, B3 and B4B.

The matrixes can be produced by solubilizing at least one sterol in a solvent, adding the saponin or saponins, and possibly the other adjuvants and lipids, whereafter the solvent might be removed and the matrix transformed into a solution where its components are not soluble, e.g. a water solution. This can be done with gel filtration, ultra filtration, dialysis or electrophores. The matrices may then be purified from excess of sterol and Quil A e.g. by centrifugation through a density gradient, or gel filtration. As solvent there might be used water or the solubilizing agents or detergents mentioned below.

The only limiting factor for matrix formation to take place is the time needed in different physico-chemical environments, the major rate limiting factor being the poor solubility of the sterol, e.g. cholesterol, in water, in which the matrix forming saponins are freely soluble.

Thus it has been shown that with Quil A and cholesterol even in solid phase matrix-like formation takes place after a relatively long time, e.g. about 1 month. Cholesterol must be brought into contact with Quil A or its purified components. If the cholesterol is brought into colloidal water suspension through treatment by ultrasonication and treatment by ultra-turrax, matrix is formed with Quil A after about 12 hours.

Consequently, any other substance such as a detergent added to the water, and which will increase the solubility of cholesterol in the aqueous medium, will decrease the time needed for the formation of matrix. It is thus possible to produce a matrix from cholesterol, water and Quil A or the subcomponents thereof, if the cholesterol is brought to a colloidal form. It is, however, more practical to add a detergent or a solvent.

Preferably the saponins are used from a concentration of at least their critical micelle formation concentration (CMC). For Quil A this implies a concentration of at least 0,03 % by weight.

As solubilizing agent there can be used detergents such as non-ionic, ionic i.e. cationic or anionic or Zwitter-ionic detergent such as Zwittergent or detergent based on gallic acid which is used in excess. Typical examples of suitable non-ionic detergents are N-alkanoyl-N-alkyl-glucamines, polyglycol esters and polyglycol ethers with aliphatic or aralylphatic acids and alcohols. Examples of these are alkylpolyoxyethylene ethers with the general formula $C_nH_{2n+1}(OCH_2CH_2)_xOH$, shortened to C_nE_x; alkyl-phenyl polyoxiethylene ethers containing a phenyl ring between the alkyl group and the polyoxyethylene chain, abbreviated $C_n\Phi E_x$, e.g. Triton X-100 = tert.-$C_8E_{9,6}$ (octyl-phenolether of polyethylene oxide), acylpolyoxyethylene esters; acylpolyoxyethylene sorbitane esters, abbreviated C_n sorbitane E_x, e.g. Tween 20, Tween 80, β-D-alkylglucos-ides, e.g. β-D-octylglucoside. Typical examples of suitable ionic detergents are gallic acid detergents such as e.g. cholic acid, desoxycholate, cholate and CTAB (cetyltriammonium bromide). Even conjugated detergents such as e.g. taurodeoxy-cholate, glycodeoxicholate and glycocholate can be used. Other possible solubilizing agents are lysolecitin and synthetic lysophosphoilipids. Even mixtures of the above-mentioned detergents can be used. When using the dialysis method the detergents should be dialysable in not too long time.

Some surface active substances greatly facilitate matrix formation. These include the intrinsic biological membrane lipids with a polar head group and a non-polar aliphatic chain e.g. phosphatidyl choline (negatively charged) and phosphat-idyl ethanolamine (positively charged).

Solubilizing can also be performed with alcohols, organic solvents or small amphiphatic molecyles such as heptane-1,2,3--triol, hexane-1,2,3-triol or caotrophic substances, acetic acid, such as trifluoro-acetic acid, trichloro-acetic acid, urea or guanidine hydrochloride.

Preferably to be used are ethyl alcohol, dioxane, ether, chloroform, acetone, benzene, acetic acid, carbon disulphid, MEGA-10 (N-decanoyl-N-methyl glucamine) and β-octylglucoside.

Various yields of matrix can be obtained with these substances, and the overall picture is that more matrix is formed the higher the concentration of the detergent is in the system.

It is technically possible to produce, purify, and sterilize matrix in any of the systems described. Therefore the adjuvant active technical preparations of matrix may contain solubilizing agents if their chemical nature and their concentration is acceptable in the final product, e.g. for vaccine purposes. However, in many cases it will be necessary to remove the solubilizing agent from the matrix by dialysis, ultrafiltration or column chromatographic techniques. It is even possible to dilute the preparation until an allowed concentration of a given solubilizing agent or detergent is reached. The preparation is diluted with water or a physiologically acceptable solution preferably to a concentration below the CMC for the solubilizing agent or detergent in the system (the preparation) used.

The solubilizing agent might be incorporated in the matrix in a molar ratio of sterol; saponin; further lipid adjuvants or solubilizing agent 1:1:1, i.e. molar ratio of the sum of lipid, adjuvants and solubilizing agent is up to half the molar that of saponin and sterol.

The solubilizing agent might alternatively be left mixed with the iscom matrix.

In order to be integrated the solubilizing agent and other immunomodulating components, should have at least one hydrophobic region. If not present such hydrophobic regions can be coupled to the components before the matrix is made.

Examples of adjuvants that can be incorporated in iscom matrix are any adjuvant, natural or synthetic, with desired immunomodulatory effect, e.g. muramyl dipeptide (MDP)-derivatives, such as fatty acid, substituted MDP, threonyl analogs of MDP; amphipatic copolymers, aliphatic amines such as avridine or DDA, poly anions such as Dextran sulphate, lipopolysaccarides such as saponins (other than Quil A). ("Future prospects for vaccine adjuvants", Warren, H.S. (1988) CRC Crit. Rev. Immunol. 8:2, 83-101; "Characterization of a nontoxic monophosphoryl lipid A", (1987) Johnson, A.G. et al, Rev. Infect. Dis. 9:5, 5512-5516; "Developmental status of synthetic immunomodulators", Berendt, M.J. et al (1985), Year Immunol. 193-201; "Immunopotentiating conjugates", Stewart--Tull, D.E., Vaccine, 85, 3:1, 40-44).

These four references are hereby incorporated as references.

The following zwitterionic, neutral, positive and negative detergents are examples of detergents that have immunomodulating, especially adjuvant activity:

Nonionic block polymer surfactants containing hydrophilic polyoxyethylene (POE) and hydrophobic polyoxypropylene (POP) that differed in mol weight percentage of POE and mode of linkage POP to POE (BASF Wyandotte Corp.), such as L72, L81, L92, pluronic L101, L121, 2531 and 31R1; octablocks T1501; B-D-octylglucosid; catjonic surfactants such as dimethyldioctadecylammonium bromide (DDA), octadecylamine (OCT), and cetyltrimethylammonium bromide (CTAB); maltostose tetrapalmitate, trehalose monomycolate, trehalose dibehenylbehenate; zwittergent detergents (N-alkyl-N,N-dimethyl-

-ammonio-3-propanesulphonate) Z3-8, Z3-10, Z3-12, Z3-14, Z3-16, obtained from Calbiochem (La Jolla, CA, USA); Z3-18 obtained from Serva (Heidelberg, FRG), Myrj 45, Brij 52, Brij 58 (also from Serva), and dioctylsulphosuccinate and Tween 20, Tween 80, Triton X-100 and sodium deoxycholate.

These detergents can be used as both detergents and adjuvants and be incorporated in the iscom matrix.

The following are examples of immunosuppressive agents that can be incorporated in a sterol (preferably cholesterol) B4B matrix: cyclosporin A, diodecyl dimethyl ammonium bromide, cationic single chain amphiphiles with more than 10 carbon atoms and preferably more than 15 carbon atoms, double chain amphiphiles with up to 14 carbon atoms, preferably up to 12 carbon atoms.

In the case a desired adjuvant or immunosuppressive agent do not have suitable hydrophobic properties, it has to be modified to comprise a hydrophobic domain for incorporation into the matrix.

The hydrophobic group that can be coupled to non-hydrophobic adjuvants are straight, branched, saturated or unsaturated aliphatic chains having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 25 carbon atoms, such as lipids, preferably 6, 7 and 8 carbon atoms; small peptides with 1, 2, 3, 4 or 5 amino acids, preferably 2, 3, 4, selected from Trp, Ile, Phe, Pro, Tyr, Leu, Var, especially Tyr; choline acid, ursodesoxycholine acid or cholesterol derivatives.

These hydrophobic groups must be bonded to a group that can be coupled to the non-hydrophobic protein such as carboxyl-, amino-, disulphide-, hydroxyl-, sulphydryl- and carbonyl group, such as aldehyde groups.

As hydrophobic groups that can be coupled are selected preferably carboxyl, aldehyde, amino, hydroxyl, and disulphide derivatives of methane, ethane, propane, butane, hexane, heptane, octane and peptides containing Cys, Asp, Glu, Lys, preferably octanal and Tyr.Tyr.Tyr-Cys, -Asp or -Glu. The hydrophobic groups with a group that can be coupled must be dissolved in water with the aid of for example the solubilizing agents and detergents mentioned above or hydrochloric acid, acetic acid, 67% by volume acetic acid, caustic liquor, ammonia, depending on what substance is to be dissolved. pH is then adjusted to the neutral direction without the substance precipitating; here it is to make sure that there is not obtained a pH-value that denaturates the protein to which the hydrophobic group is to be coupled.

Hydrophobic groups with a carboxyl group as coupling molecule can be coupled to the adjuvants through water-soluble carbodiimides or composite anhydrides. In the first case the carboxyl group is activated at pH 5 with carbodiimide and mixed with the protein dissolved in buffer pH 8 with a high phosphate content. In the latter case the carboxy compound is reacted with isobutylchloroformate in the presence of triethylamine in dioxane or acetonitrile, and the resulting anhydride is added to th protein at pH 8 to 9. It is also possible to convert the carboxyl group with hydrazine to hydrazide which together with aldehydes and ketones in periodate-oxidized sugar units in the protein gives hydrazone bonds.

The amino groups with nitrous acid can at low temperature be converted to diazonium salts, which gives azo bonds with Tyr, His and Lys.

The hydroxyl groups with succinic anhydride can be converted to hemisuccinate derivatives which can be coupled as carboxyl groups.

Aldehyde groups can be reacted with amino groups in the protein to a Schiff's base.

Several coupling groups and methods are described in Journal of Immunological Methods, 59 (1983) 129-143, 289-299, Methods in Enzymoloy Vol 93 pp 280-33, and in Analytical Biochemistry 116, 402-407 (1981) which are here incorporated as references.

The lipids other than sterol can be fats or fat resembling substances such as triglycerides or mixed triglycerides containing fatty acids with up to 50 carbon acids such as saturated fatty acids with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30 carbon atoms e.g. butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid, or unsaturated fatty acids with up to 30 carbon atoms, such as hexadecene acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid; hydroxy-fatty acids such as 9,10-dihydroxy stearic acid, unsaturated hydroxy fatty acids such as castor oil, branched fatty acids; glycerol ethers, waxes i.e. esters between higher fatty acids and monohydric alcohols; phospholipides such as derivatives of glycerol phosphates such as derivatives of phosphatidic acids i.e. lecithin, cephalin, inositol phosphatides, spingosine derivatives with 14, 15, 16, 17, 18, 19 and 20 carbon atoms; glycolipids isoprenoids, sulpholipids, carotenoids, steroids, sterols, cholestanol, caprostanol, phytosterols, e.g. stigmasterol, sitosterol, mycosterols, e.g. ergosterol, bile acids e.g. cholic acid, deoxycholic acid, chenodeoxycholic acid, litocholic acid, steroid glycosides, esters of vitamine A, or mixtures thereof.

These and other useful lipids are described in: Lipid biochemistry and introduction, Ed. M.I. Gurr, A.I. James, 1980, Chapman and Hall, London, New York, University Press Cambridge, which hereby is incorporated as a reference.

Preferably cholesterol phosphatidylcholine, liposomes or intralipid® (Oleum soya fractionate 200 g, Lechitinum fractionate vitello ovi 12 g, glycerol 22.5 g, and H_2O up to 1 liter) are used.

The lipids can be added at any stage in the process, preferably before the addition of the saponin but lipids could also be added after the saponin.

The matrix is best produced by the dialysis method as follows.

Cholesterol dissolved in 20% MEGA-10 or any other suitable detergent, preferably a detergent that can be removed by dialysis, e.g. β-octylglucoside, (in H_2O or a suitable buffer) is mixed with 5 times as much Quil A (solid or dissolved in water or a suitable buffer, e.g. PBS). The mixture is dialysed extensively against PBS, first over night at room temperature (because MEGA-10 will precipitate at +4°C), then at +4°C. The matrixes are purified from excess Quil A and cholesterol by pelleting through e.g. 30% (w/w) sucrose (e.g., a TST 41.13 rotor 18 h, 39.000 rpm, 10°C). The pelleted matrixes are dissolved in PBS (or any other suitable buffer) and the concentration adjusted to 1 mg/ml).

The present matrix can be used as an immunomodulating substance. It can be used as a potentiating agent for an immunosuppressive substance or an adjuvant, either mixed therewith or integrated in the matrix.

A matrix containing a sterol such as cholesterol, saponins, adjuvants and optionally further lipids can be used as an adjuvant. It can be used for potentiating the antigenic effect of any antigen or antigenic determinants from any pathogenic organism or any fragments or subunits of, or derived from these. Thus it can be used as an adjuvant for those antigens that are integrated in an iscom. Such antigens are mentioned

in the EPC-patent applications 83850273.0 and 85850326.1, which are hereby incorporated as references. Thus the matrix can be used as adjuvants together with antigenes or antigenic determinants derived from viruses with or without envelope, bacteria, protozoa, mycoplasmas, helminths, mollusca or together with such whole organisms. The antigenes or antigenic determinants might further be hormones, enzymes, carbohydrates and carbohydrate-containing structures such as lipopolysaccharides, peptides or proteins or recombinants thereof.

The present invention thus also covers human or veterinary medicine, characterized in that it comprises at least one matrix and one or more antigenic or immunosuppressive substances and a pharmaceutically acceptable vehicle in mixture or in separate compartments.

The invention also concerns a vaccine comprising an matrix, one or more antigens and a pharmaceutically acceptable vehicle.

Further the invention concerns a kit comprising such a medicine or vaccine.

In some medicines or vaccines the detergent used when preparing the matrix can be present if the detergent is allowed for the product in question.

The effect of the new adjuvant complex according to the invention will now be described in immunostimulating experiments.

1. Comparison between the immunogenic effects from antigens presentated as iscoms, micelles or micellas plus the new matrix.

Mice were immunized with envelope protein from influenza virus in the form of iscom complex, micelles and micellas togehter with the new complex according to the invention (so called

matrix). The immune response was evaluated by measuring the antibodies with ELISA technique 15, 30, 44 and 50 days after injection. The following injections were made:

1. 5 µg Micell + 0,1 µg matrix were mixed and injected in the left foreleg.

2. 5 µg Micell + 0,1 µg matrix injected separately in the right and left foreleg respectively.

3. 5 µg Micell

4. 5 µg iscom prepared according to EPC 83850273.0

TABLE 1

DAY	1	2	3	4
15	23.700 ± 9.500	12.900 ± 14.500	18.900 ± 9.500	41.600 ± 1.000
30	30.800 ± 10.500	8.800 ± 7.100	9.800 ± 3.600	80.700 ± 21.700
44	30.000 ± 17.600	32.600 ± 17.300	17.900 ± 4.200	129.100 ± 78.400
50	309.300 ± 89.000	136.700 ±103.700	87.600 ± 18.200	880.430 ±295.500

No side effects in the form of local reactions were noted.

From this experiment one can conclude that envelope protein from influenza in the form of iscom or micelles plus matrix gives the highest antibody titres. Matrix can be presented in a very low dose and still have adjuvant effect. In order to get an adjuvant effect in mice, Quil A in free form is required in a dose a 100 times the dose of matrix, i.e. 10 µg. With that dose Quil A begins to give local side reactions. On order for matrix to have an obvious adjuvant

effect the antigen in multimeric form should be injected in the same region e.g. leg as the matrix, i.e. the injected adjuvant matrix complex and antigen should be presented in a region, that is drained to the same lymphatic gland.

2. Comparison between the immunogenic effects from envelope protein from influenza in the from of iscom or micelle with or without matrix or difteritoxoid (DT).

Mice were injected with envelope protein in the following forms:

1. 5 µg Iscom + 5 µg DT

2. 5 µg Iscom

3. 5 µg micelle

4. 5 µg micelle + 0,1 µg matrix

The antibody response in envelope proteins was estimated in the serum with ELISA-technique.The following results were obtained:

TABLE 2

DAY	1	2	3	4
15	52.800 [1)]	48.300	8.400	29.000
30	119.202	155.567	22.107	87.000
50	110.600	136.200	33.400	96.500
65	1.691.000	3.783.000	283.300	1.149.000
80	562.800	2.529.000	512.300	976.500

1) ELISA-titer where the last dilution gives a significant positive value at 450 nm.

No visible side effects in form of local reactions could be noted.

One can conclude that envelope protein from influenza virus in the form of iscom or micelles plus the adjuvant complex (matrix) according to the invention gives the highest antibody titres. The dose of matrix can be kept very low, i.e. 0,1 µg, and still has a notable adjuvant effect.

3. Comparison between the immunogenic effects from diphtheria toxoid (DT) in monomeric form, monomeric DT + iscom containing envelope protein from influenza virus, monomeric DT in mixture with Quil A and cholesterol and monomeric DT + adjuvant complex (matrix) according to the invention.

In this experiment diphtheria toxoid is used as a model antigen in monomeric form.

Mice were injected with diphtheria toxoid in the following forms:

1. 5 µg DT (diphtheria toxoid)

2. 5 µg DT + 5 µg iscom

3. 5 µg DT+ 0,5 µg Quil A + 0,1 µg CL (cholesterol)

4. 5 ug DT + 0,1 ug matrix

TABLE 3

DAY	1	2	3	4
15	≤ 30	≤ 30	≤ 30	≤ 30
30	≤ 30	≤ 30	≤ 30	≤ 30
50	≤ 30	≤ 30	≤ 30	≤ 30
65	≤ 30	90	≤ 30	10.000
80	≤ 30	60	90	1.100

The immungenic response to DT is low in all the groups. The

best result is obtained with mice immunized with diphtheria toxoid plus matrix according to the invention.

From the experiments above one can conclude that the best results are obtained when the matrix according to the invention is used together with the antigen in multimeric form. The matrix according to the invention has thus proved to give very good results as adjuvant compared with e.g. Quil A in free form. Thus it is worth noting that Quil A is effective as adjuvant in free form in doses such as 10 µg for mice, 50 µg for guinea-pigs and 1 mg for cattles. A practical volume for injection of a vaccine is 1 ml for small animals and 2 to 5 or 10 ml for big animals. As CMC (the critical micelle concentration) for Quil A is 0,03%, 1 ml will imply an amount of 300 µg when 1 ml is injected. After injection, however, due to the dilution effect, the concentration will become lower than CMC and the micelle will become unstable.

According to the present invention, however, the saponin and especially the Quil A molecules will be bounded together with cholesterol molecules so that a relatively stable complex is formed at very low concentrations. This complex is effective as adjuvant in a dose, which corresponds to 0,1 µg Quil A, i.e. 100 times lower than when Quil A is presented in free form.

The Figures show:
Fig. 1 shows an electron microscope picture of a typical matrix;
Fig. 2 shows U.V. eluation profiles for subfractions of Quil A;
Fig. 3 demonstrates HPTLC-separation of Quil A and its subfractions;
Fig. 4 shows FAB-mass-spectra for the new substances according to the invention;
Fig.:s 5 and 6 show ^{13}C-NMR-spectra (2-regions) for the new substances;

Fig.:s 7, 8 and 9 show complete ^{13}C-NMR-spectra for B2, B3 and B4B, respectively;
Fig. 10 shows the β-amyrin-skeleton;
Fig.:s 11 and 12 show the ^{1}H NMR-spectra for the new substances;
Fig. 13 shows parts of the spectra in Fig.:s 7 and 8; and
Fig. 14 shows a 2-dimensional NMR-spectrum for substance B3.

The invention will now be described further with the following example.

Example 1:

Matrix (Cholesterol-Quil A complex)

1 mg of cholesterol dissolved in 20 % MEGA-10 (in H_2O) was mixed with 5 mg of solid Quil A. The Quil A was dissolved and the mixture was dialysed extensively against PBS, first over night at room temperature, then at +4°C. The iscom matrixes were purified from excess Quil A and cholesterol by pelleting through 30 % (w/w) sucrose (TST 41.13 rotor 18 h, 39.000 rpm, 10°C). The pelleted matrixes were dissolved in PBS and the concentration adjusted to 1 mg/ml (traced by a small amount of ^{3}H-cholesterol).

Example 2:

MDP (muramyldipeptide, Sigma, adjuvant peptide) was conjugated to phosphatidyl ethanolamine (PEA) using N-ethyl-N'-(dimethyl-aminopropyl) carbodiimide hydrochloride as described by Lefrancier et al., 1977 (Lefrancier, P., Choay, J., Derrien, M. and Lederman, I. (1977) Int. J. peptide Protein Res. 9:249--257).

To 1 mg of cholesterol (in 20% MEGA-10 in H_2O) was added an equimolar amount of MDP-PEA (in MEGA-10 or DMSO or any other water micible solvent), an equimolar amount of phosphatidyl choline and 7 mg of Quil A (a slight excess in comparison to 5 mg that is required for IM-formation). After a short in-

cubation at room temperature (15-30 min) the mixture was extensively dialysed against PBS (room temperature 4-12 h, then at +4°C).

After completed dialysis, the matrix-complexes with the additional adjuvant integrated were purified from excess Quil A by pelleting through 10% sucrose.

Example 3:

To 1 mg of cholesterol (in 20% MEGA-10 in H_2O) was added an equimolar amount of Avridine (N,N-dioctadecyl-N'N'-bis(2--hydroxyethyl)propenediamine (in MEGA-10 or DMSO or any other water micible solvent), an equimolar amount of phosphatidyl choline and 7 mg of Quil A (a slight excess in comparison to 5 mg that is required for IM-formation). After a short incubation at room temperature (15-30 min) the mixture was extensively dialysed against PBS (room temperature 4-12 h, then at +4°C).

After completed dialysis, the matrix-complexes with the additional adjuvant integrated were purified from excess Quil A and adjuvant by pelleting through 10% sucrose (the same method as described on page 14, last paragraph).

Example 4:

To 1 mg of cholesterol (in 20% MEGA-10 in H_2O) was added an equimolar amount of DDA (dimethyl dioctadecyl ammonium bromide (in MEGA-10 or DMSO or any other water micible solvent), an equimolar amount of phosphatidyl choline and 7 mg of Quil A (a slight excess in comparison to 5 mg that is required for matrix-formation). After a short incubation at room temperature (15-30 min) the mixture was extensively dialysed against PBS (room temperature 4-12 h, then at +4°C).

After completed dialysis, the matrix-complexes with the additional adjuvant integrated were purified from excess Quil A

and adjuvant by pelleting through 10% sucrose (the same method as described on page 14, last paragraph).

Example 5:

2 g of Mega 10 is added to 10 ml of water before the addition of 200 mg cholesterol, and the cholesterol is dispersed by ultrasonication/ultraturrax. As much as 1.6 ml of this mixture can be added to the 10 ml of 2% Quil A-solution. The reaction mixture clarifies completely after less than one hour indicating that all the cholesterol has been reacted. It can be seen in the electron microscope that the concentration of matrix is very high even if the concentration of detergent in this case is 10%. Removal of the detergent by dialysis or ultrafiltration does not quantitatively affect the number of matrix particles, and the solution of matrix stays completely clear.

This experiment indicates that matrix formation takes place when the surfactants are present in the reaction mixtures, and that complete matrix formation takes place in very high concentrations of detergent.

Example 6:

Preparation of the Quil A components B2, B3 and B4B according to the invention.

5 g Cortex quillajae (Nordiske Droge of Kemikalieforretning, Copenhagen, Batch nr 8372) and 50 ml destillated water was mixed by a magnetical stirrer for 3 hours at room temperature. The liquid phase was separated through a Büchner funnel by a filter paper and was purified by filtering through a Metricel Gelman membrane 0.22 µ. Such an extract contains 2.5% dry material.

The crude extract was dialysed against 200 volumes of destillated water in a Visking-tube without weld 20/32 for 48 hours with exchange of water after 24 hours. This extract is called DQ.

The dialysed extract above was subjected to ion exchange chromatography. A column of DEAE-cellulose equilibrated with 0.1M Tris-HCl pH 7.5 was prepared (Whatman DE52) in a K 9/15 column (Pharmacia Fine Chemicals). The bed material was equilibrated with 0.1M Tris-HCl buffer pH 7.5. The column was eluted either stepwise or by a linear salt gradient at a flow rate of 60 ml/h using a peristaltic pump. 50 ml DQ was introduced on the column and 300 drop (equivalent to approx. 5 ml) fractions were collected. Under these conditions, some of the substances in DQ passed unbound through the column, as will be seen from Fig. 2A (peak A). Elution was continued until no UV absorption was detectable. The absorption of the effluent liquid was recorded at 280 nm by a Uvicord II system (LKB-Produkter), and fractions were collected by a Golden Retriever (ISCO). At this point a buffer containing 0.2M NaCl made up in start buffer was introduced. As can be seen in Fig. 2A, a peak B is eluted. However, some substances were still attached to the bed material to such a degree that elution was difficult even with concentrated NaCl. These substances were the ones that contributed to the brownish colour of DQ, whereas peak A and B were only slighly coloured or completely colourless, respectively. In the next purification step, peak B was pooled and subjected to gel exclusion chromatography on Sephadex G50 fine equilibrated with M/50 phosphate buffer pH 7.5 in a K 16/70 column eluted at a flow rate of 10 ml/h. Desalting was carried out on Sephadex G25 medium in a K 16/40 column. Elution was carried out using a hydrostatic head of 50 cm. As will be seen from Fig. 2B, the UV profile showed 3 peaks. Peak C was eluted in the void volume (as determined by Blue Dextran 2000, Pharmacia Fine Chemicals) and peak E was eluted in the total volume of the column (also determined by potassium chromate). Peak D was well separated from peak C and E, but as can be seen in Fig. 2B, the presence of a shoulder indicated that peak D consisted of at least two substances.

Consequently, peak D was pooled and subjected to a new separation on DEAE-cellulose. The starting conditions were the same as in the first ion exchange experiment, and as a result all the material was adsorbed to the column. Elution was now continued with a linear NaCl gradient increasing from 0 to 1 molar (made up in the start buffer) in the course of 300 ml. The result of this experiment is shown in Fig. 2C. Two peaks F and G appeared in the UV profile. F was clearly separated and a single substance (section 3.3), but peak G could not be isolated since it was contaminated with F. In order to investigate the homogeneity of peak F, it was pooled, desalted on Sephadex G25, and rechromatographed in an identical experiment. As can be seen from Fig. 2D, only one symmetrical peak (H) appeared at the position of peak F.

Anyone of the fractions (also the DQ-extract) can be further purified as follows.

Fraction H was lyophilized and dissolved in chloroform/ /methanol/H_2O (60:40:9, v/v/v). 200 mg was then applied to an HPLC column (4.5x50 cm) packed with silicic acid, Iatrobeads RS-8060 (Iatron Labs, Tokyo, Japan). A pump speed of 5 ml/min was used for pumping a total of 2 L solvent, collecting 200 fractions of 10 ml with a gradient of chloroform/ /methanol/H_2O (60:40:9 to 50:40:10). The fractions were analyzed with thin layer chromatography in the following way. 2 µl of every second fraction was analyzed by developing thin layer liquid chromatography plates (TLC-plates) (HPTLC, Merek, Bodman Chemicals, Gibbstown, NJ) in chloroform/ /methanol/0.2% $CaCl_2$ (50:40:10 v/v/v) and the glycosides were determined by being greencoloured with anisaldehyde reagent (acetic acid/sulphoric acid/paraanisaldehyde (98:2:1)). The Quil A starting material was used as a reference of the R_f-value. (See Fig. 3, which shows a HPTLC-separation of: lane 1, Quil A (fraction H); lane 2, B1; lane 3, B2; lane 4; B3; lane 5, B4A; and lane 6, B4B).

Fractions that comigrate with B2, B3 and B4B having identical R_f-values were pooled and analyzed for purity with TLC. These crude fractions usually must be chromatographed two times in order to become pure enough. Fractions B1 and B4A are inactive and therefore are not separated further.

The thus enriched components were further purified on an HPLC column (21.2x250 mm) packed with 5 µ spherical silica particles (Zorbax Si, DuPont, Wilmington, DE). 40 mg enriched fraction B2, B3 or B4B dissolved in 1 ml chloroform/methanol/ /H_2O (60;40;9 v/v/v) was put on the column. A pump speed of 3 ml/min was used for pumping a total of 0.9 l solvent, collecting 300 fractions of 3 ml with a gradient of chloroform/ /methanol/H_2O (60;40;9 to 50;40;10 v/v/v). Fractions were analyzed on glass-backed HPLTC-plates as above. Purified fractions were pooled and evaporated to dryness in a rotary evaporator <30°C, dessiccated and stored in <-20°C. Approximately 20-25 rounds of this purification step was used i.e. using (20-25)x200 mg = 4-5 g Quil A starting material, including rechromatography to prepare 1 g of fraction B3. The yield of B2 and B4B was about 40% of the yield of B3.

The so prepared components B2, B3 and B4B were analyzed as follows.

a) Mass Spectrometry

Negative FAB-MS, Fig. 4, and positive FAB-MS (data not shown) were carried out for determination of molecular weights of the purified Quil A ocmponents B2, B3, B4A, and B4B. The data shown in Fig. 4 are preliminary and will have to be re--acquired in a neutral pH matrix such as glycerol rather than in triethanolamine which was used for the spectra shown in Fig. 4. This is necessary because extreme alkali-lability of the compounds, pH > 8.5 have been demonstrated. Peaks at m/z 595, 744, and 893 stem from the matrix triethanolamine and

should be disregarded. Our fraction B4A, which does not have any adjuvant or ISCOM particle forming capacity, seem to be identical with that described by Komori et al (for structure, see Fig. 10). The peaks corresponding to molecular weights of the three thus far most interesting glycosides are at: m/z 1988, B2; m/z 2150, B3; and m/z 1862, B4B.

b) ^{13}C-NMR

Fig.:s 5 and 6 show two regions, aliphatic carbon (8-45 ppm) and anomeric carbon (90-115 ppm), respectively, of the ^{13}C-NMR spectra for the full size fractions: A, B2 (20 mg); B, B3 (80 mg); and C, B4B (40 mg). All spectra were obtained in the solvent-system, chloroform/methanol/water (30:60:8, v/v/v). The triterpenoid region is well resolved (8-45 ppm, Fig. 6) and has been partially assigned as seen in Table 4.

TABLE 4

Partial ^{13}C-NMR signal assignment (ppm) for β-amyrin five-ring segment of fractions B2, B3, and B4B (see Fig. 5) obtained in chloroform/methanol/water (30:60:8, v/v/v).

Carbon#	B2	B3	B4B	Reference
C9[a]	-[b]	-[b]	-[b]	45.5
C10	36.4	36.2	36.3	37.0
C12	122.5	122.2	122.5	123.1
C13	144.1	146.6	143.6	144.8
C14	41.5	41.8	41.9	41.6
C15	30.0	30.5	30.7	30.7
C18	43.0	42.8	41.9	42.7
C20	30.7	30.6	30.6	30.7
C25	16.0	16.1	16.0	15.9
C26	17.7	17.3	17.6	17.6
C29	32.9	32.9	32.9	32.2

[a] Numbered as in Fig. 5.

[b] Hidden under methanol signal of solvent (confirmed with a DEPT experiment).

These assignments have been performed from studying a large number of reference-spectra obtained in various solvents and by analyzing the signals that are solvent-independent by a statistical comparison (data not shown). Fig. 6 shows the region between 80-148 ppm in the spectra of the three compounds, A, B2; B, B3; and C, B4B, featuring two double-bond carbon signals at 122 and 143 ppm corresponding to C-12 and C-13, respectively, in the β-amyrin skeleton (Fig. 10). The anomeric-carbon region, between 90-115 ppm, shows the presence of approximately 9-10 signals corresponding to the same amount of sugar-residues in the compounds.

Conclusion: Structural differences can be identified between fractions: A, B2; B, B3; and C, B4B, in both spectral regions corresponding to mainly the triterpenoid region and the oligosaccharide portions of the molecules, respectively. The exact amounts of sugars can not be determined at this point.

c) ^{1}H NMR

Fig. 11 demonstrates the full proton spectrum (0-10 ppm) and Fig. 12 partial proton spectrum (anomeric region, 4.0--6.0 ppm), respectively, of fractions: A, B2; B, B3; and C, B4B. The spectra are obtained from samples (≈ 10 mg, ≈ 600 scans) dissolved in DMSO.d_6/D_2O (98:2, v/v). To the far left in the spectrum (Fig. 11), at 9.4 ppm, the signal from the aldehyde proton on carbon-24 (see Fig. 5) is found. The doublet nature of this peak, a peak which is supposed to be a singlet, since it has no neighbouring protons to couple to, offers an explanation to the unusually complex anomeric region which is poorly resolved (as seen in the expansion in Fig. 12).

The doublet can be due to an aldehyde proton in two different compounds or to the presence of chemical exchange between two different populations of the same molecule (this process is

slow enough in NMR time scale to be observed) thus explaining the different integrals of the peaks in the doublet at different temperatures as shown in Fig. 13 and Table 5.

Table 5

Temperature in Degree K	Shift 1	Shift 2	Difference in Shifts	Integral Quote Shift 1/Shift 2
301	9.46	9.44	0.02	1.61
351	9.47	9.46	0.01	1.99
361	9.48	9.47	0.01	2.23

Fig. 13 and Table 5 (above) demonstrate that the relative integral of the peaks varies with temperature and that the two peaks move closer to each other at a higher temperature, both indicating that it can not be two different molecules but rather two different populations of the same molecule. This would explain the complex anomeric region by suggesting that many anomeric protons in the molecule would have double resonances due to different chemical environments in the two populations. However, the present set of data indicates that differences in the glycosylation of the compounds could provide part of the explanation of their structural differences (Fig. 12), by demonstrating different amount of anomeric proton signals in the spectra. The FAB-MS data for fractions B2, B3 and B4B also does not rule out the formal possibility that two similar size molecules, with very similar physico--chemical properties, exist that have the same amount of sugars but differ in linkage-positions and/or sequence.

Conclusion: In general, 1-dimensional ^{1}H NMR spectra from 8-10 sugar containing earlier unknown molecules are not sufficient for assignment of protons and detailed structural characterization. For resolving all signals and for making proper assignments through out the compounds it will be necessary to use the 2-dimensional NMR technique as well as

chemically degrade the compounds for analysis. Both homonuclear (^{1}H-^{1}H) and heteronuclear (^{1}H-^{13}H) COSY, TOCSY as well as NOESY. The 2-dimensional proton phase sensitive correlation double quantum filtered NMR spectrum (DQFPSCOSY) for fraction B3 is shown in Fig. 14.

d) Summary of Structural Data

The conclusion of data generated thus far is that the active fractions that have adjuvant activity and ISCOM particle forming capacity in Quil A contain unique glycosylated triterpenoid-saponins that differ between each other in both their triterpenoid and glycan parts. They have an approximate structure like the one described in Fig. 10 and consist of a five-ring steroid skeleton of β-amyrin type and contains 8-11 sugar residues.

Example 7

0.1 mg cholesterol was mixed with ^{3}H-cholesterol (10 mg/ml dissolved in 20% MEGA-10 in H_2O) and 0.5 mg B2 or B3 or B4B or mixtures thereof. The volume was adjusted to 0.5 ml and the mixture dialysed against PBS on a preparation treated with ammonium molybdate (negative colouring technique). The dialysed preparations were analysed for the presence of complex with iscom structure by electron microscopy (EM) and analytical gradient centrifugation. In EM the iscom structure is characterized by a cage-like particle with a diameter of 40 nm composed of subunits with annular structure with a diameter of 12 nm. For sedimentation studies the sample is placed over a sacharose gradient (10-50%) and centrifuged for 18 hours, +10°C in a TST 41,14 rotor, 40 000 rpm. The gradient is collected in 18 fractions (fraction 1 = the bottom and fraction 18 = the top). By localizing the ^{3}H-cholesterol activity in the gradient, one can tell the sedimentation constant and see if complexes have been made.

B4B forms typical iscom structures with cholesterol but has no potent adjuvant activity.

B2 does not form iscom-like structures with cholesterol but binds to cholesterol. Together with B4B, B2 forms iscom-like structures with cholesterol. B2 has a weak adjuvant activity.

B3 binds to cholesterol but not in iscom-like structures. With B4B, B3 like B2, can form iscom-like structures with cholesterol. B3 has adjuvant activity.

CLAIMS

1. A matrix which is not a lipid vesicle and without any intentional antigens or antigenic determinants comprising at least one lipid and at least one saponin for use as an immunomodulating agent.

2. A matrix according to Claim 1, comprising at least one lipid, at least one saponin and at least one adjuvant.

3. A matrix according to Claim 2, comprising a sterol, preferably cholesterol, one or more saponins, one or more adjuvants and one or more further lipids.

4. A matrix according to any of Claims 1-3, characterized in that the saponin is a triterpensaponin, especially Quil A or one or more components thereof.

5. A matrix according to Claims 1-4, comprising one or more immunomodulating compounds.

6. A matrix according to any of Claims 1-5, characterized in that it has an open spherical structure consisting of circular sub-units or parts of the spherical structure under electron microscopy and a sedimentation constant of about 12-22 S.

7. A process for preparing a matrix which is not a lipid vesicle and without any intentional antigens or antigenic determinants comprising at least one lipid and at least one saponin and optionally an adjuvant for use as an immunomodulating agent or a vaccine, characterized in that at least one lipid is solubilized in a solvent, the saponin or saponins are added, the other adjuvants and lipids are optionally also added, whereafter the solvent may be removed for example by dialysis, ultra filtration, gel filtration or eletrophoreses, or be diluted.

8. A process according to Claim 7, characterized in that the lipid is a sterol, especially cholesterol, and the saponin is a triterpensaponin, especially Quil A or one or more components thereof.

9. A process according to Claim 7 or 8, characterized in that one or more immunomodulating compounds are added.

10. A process according to Claim 7-9, characterized in that the sterol and saponin are solubilized in the lipids and/or adjuvants.

11. Glycosylated triterpenoid saponins derived from Quillaja Saponaria Molina of Beta Amyrin type with 8-11 carbohydrate moieties which have the following characteristics:

a) Substance B2 has a molecular weight of 1988, a carbon 13 nuclear magnetic resonance (NMR) spectrum as indicated on Fig.:s 5A and 6A and a proton NMR spectrum as shown in Fig.:s 11A and 12A;

b) Substance B3 has a molecular weight of 2150 and has a carbon 13 NMR spectrum as shown in Fig.:s 5B and 6B, and a proton NMR spectrum as shown in Fig.:s 11B and 12B;

c) Substance B4B has a molecular weight of 1862, a carbon 13 NMR spectrum as shown in Fig.:s 5C and 6C, and a proton NMR structure as shown in Fig.:s 11C and 12C.

12. A process for preparing glycosylated triterpenoid saponins derived from Quillaja Saponaria Molina of Beta Amyrin type with 8-11 carbohydrate moieties which have the following characteristics:

a) Substance B2 has a molecular weight of 1988, a carbon 13 nuclear magnetic resonance (NMR) spectrum as indicated on Fig.:s 5A and 6A and a proton NMR spectrum as shown in Fig.:s 11A and 12A;

b) Substance B3 has a molecular weight of 2150 and has a carbon 13 NMR spectrum as shown in Fig.:s 5B and 6B, and a proton NMR spectrum as shown in Fig.:s 11B and 12B;

c) Substance B4B has a molecular weight of 1862, a carbon 13 NMR spectrum as shown in Fig.:s 5C and 6C, and a proton NMR structure as shown in Fig.:s 11C and 12C,

characterized in extracting the bark from Quillaja Saponaria Molina with water, dialysing the aqueous extract, lyophilizing the dialysate, subjecting the lyophilized matter to gel filtration on Sephadex G-50 and subsequent weak anionic exchange chromatography on DEAE-cellulose, eluting the products with 0.2M sodium chloride at neutral (pH) conditions and separating them by repeated chromatography on silica gel.

13. A vaccine comprising a matrix as hereinbefore defined according to Claims 1-6, one or more antigens and a pharmaceutically acceptable vehicle.

14. A kit for human or veterinary medicine use, characterized in that it comprises at least one matrix according to Claims 1-6 and one or more immunomodulating substances and a pharmaceutically acceptable vehicle in mixture or in separate compartments.

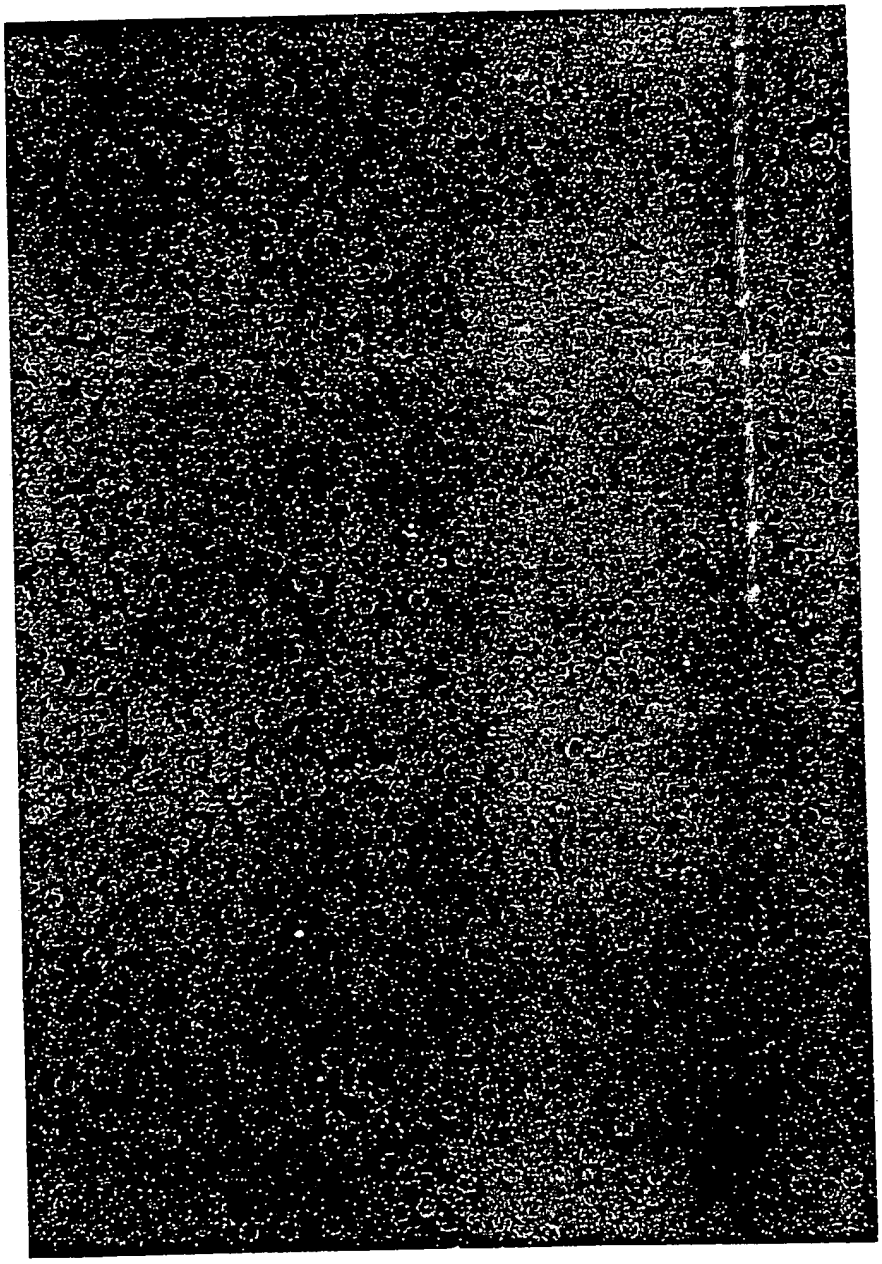


FIG.1

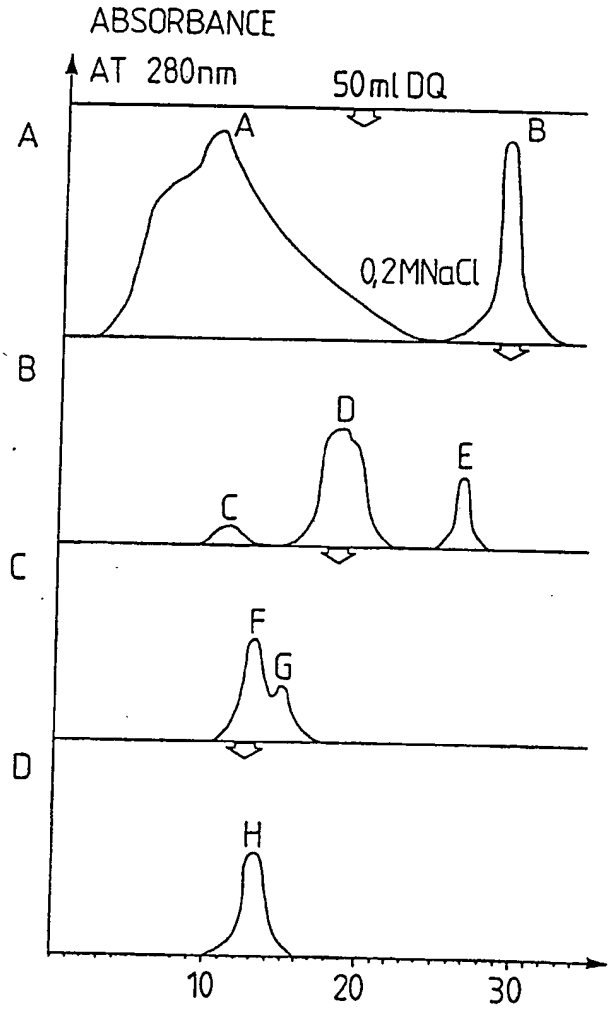


FIG.2

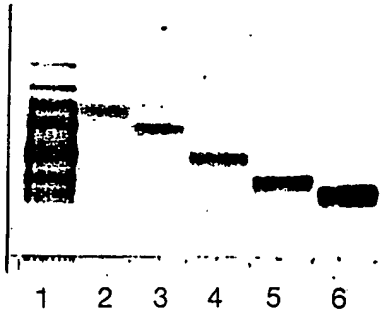


FIG. 3

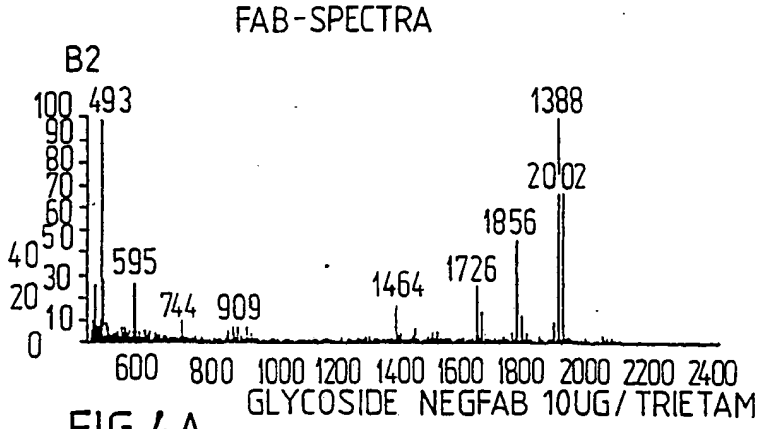


FIG.4A

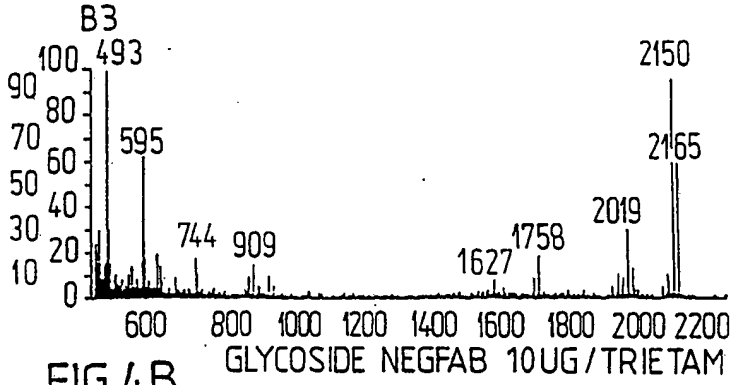


FIG.4B

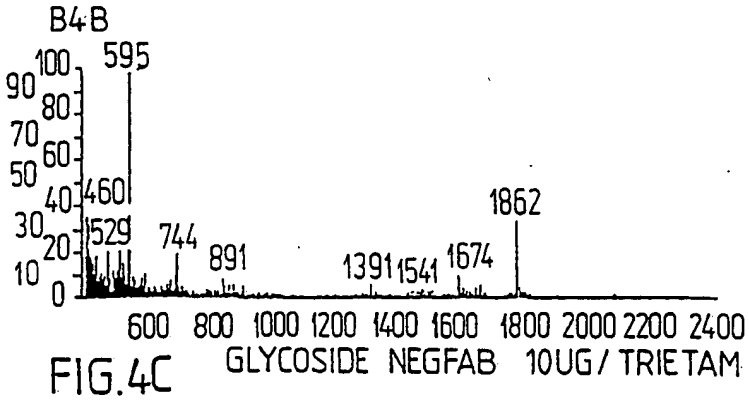


FIG.4C

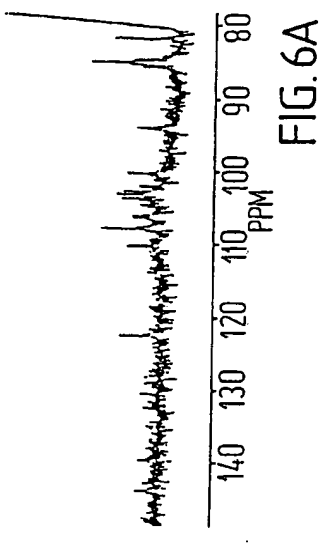


FIG. 6A

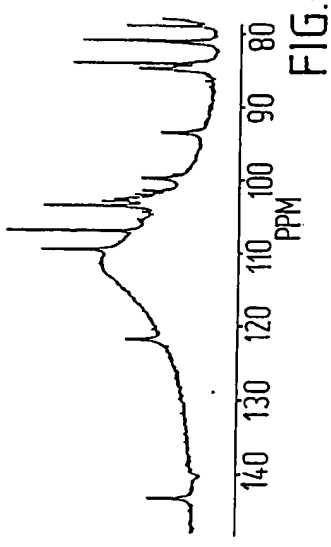


FIG.6B

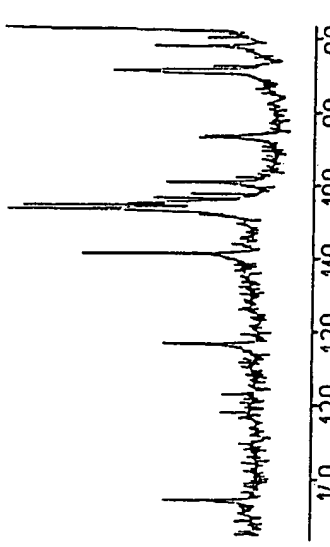


FIG.6C

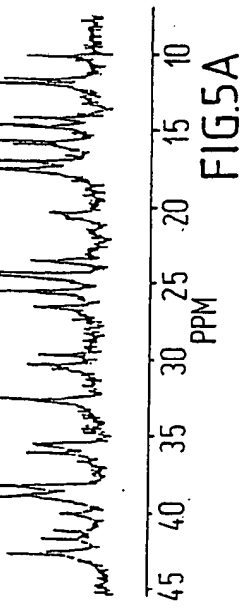


FIG.5A

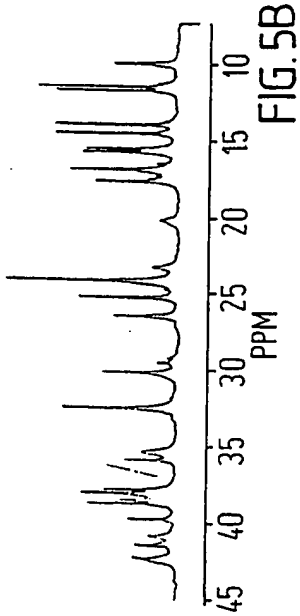


FIG.5B

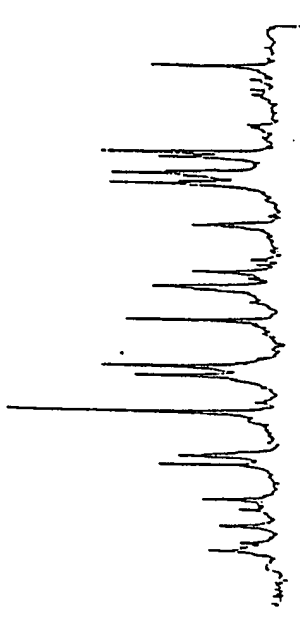
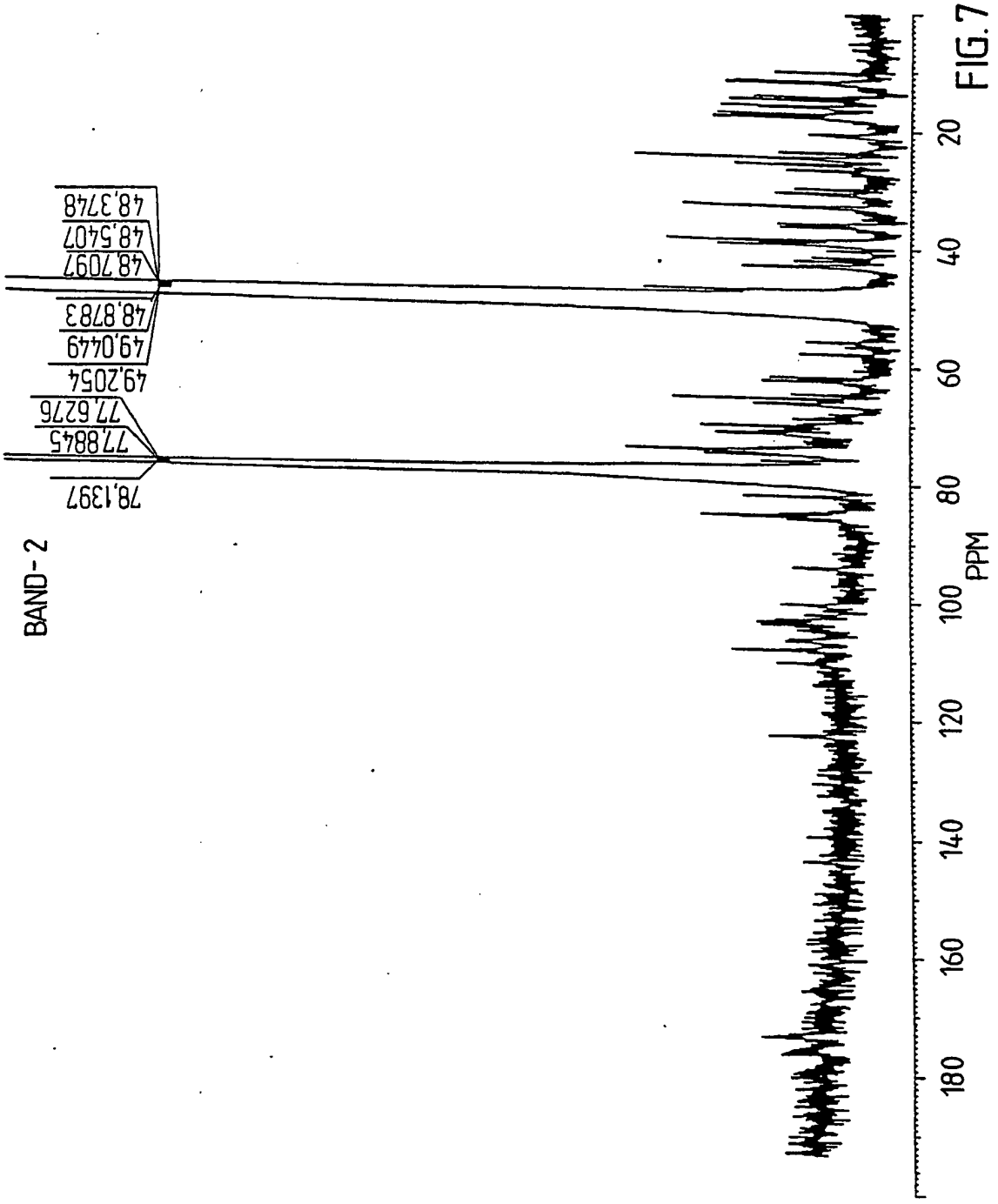


FIG.5C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PPM

FIG.7

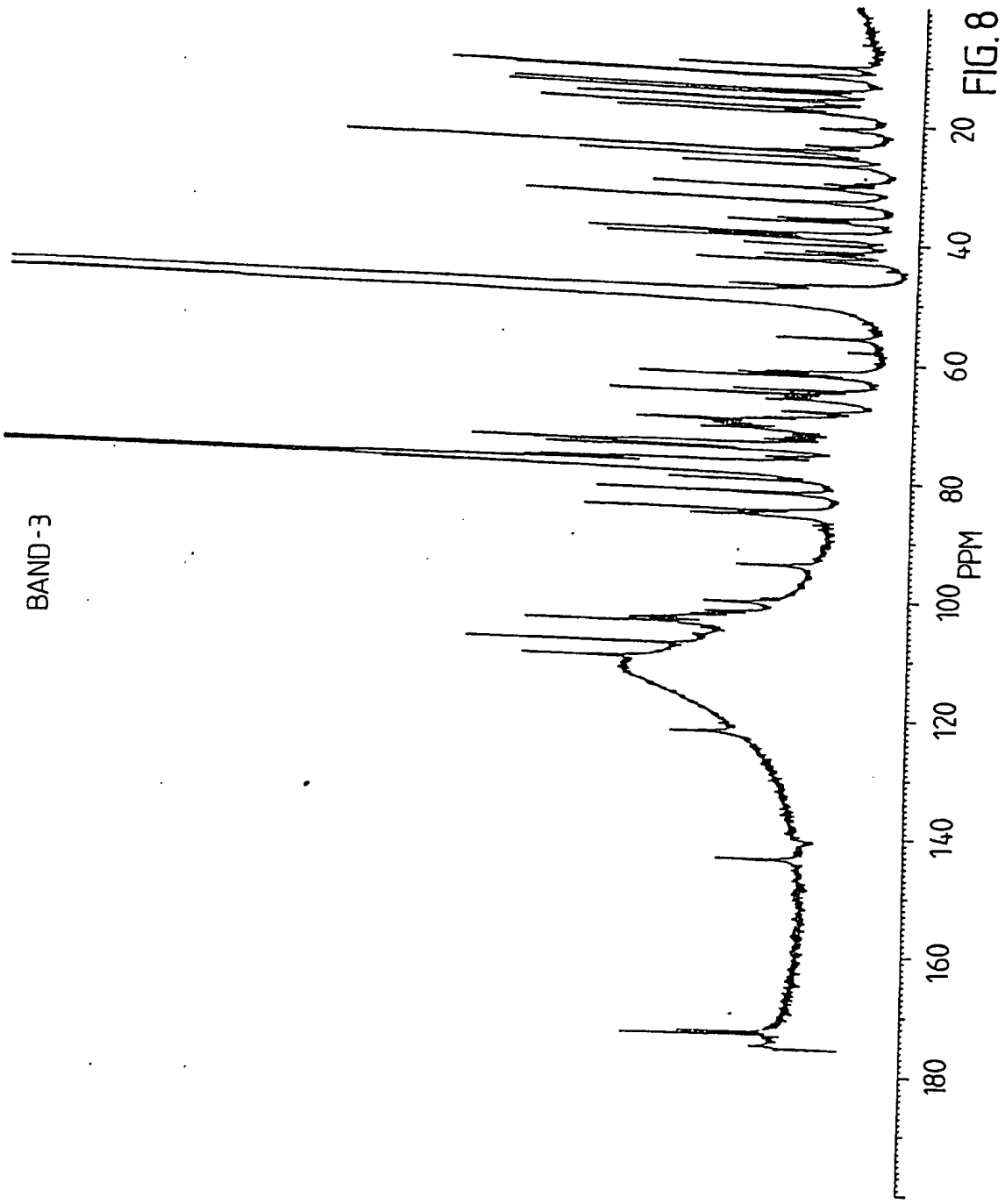

FIG. 8

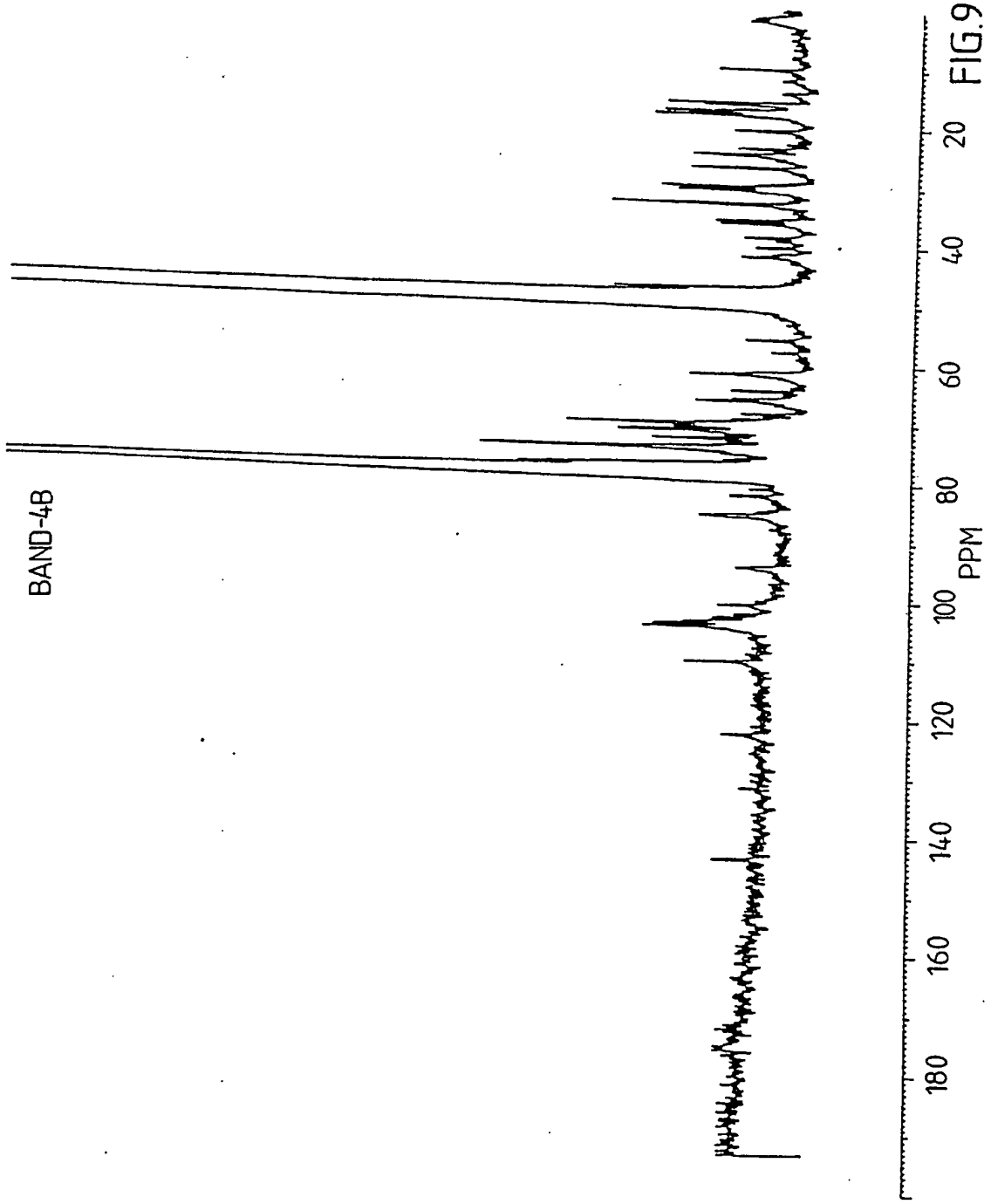

FIG.9

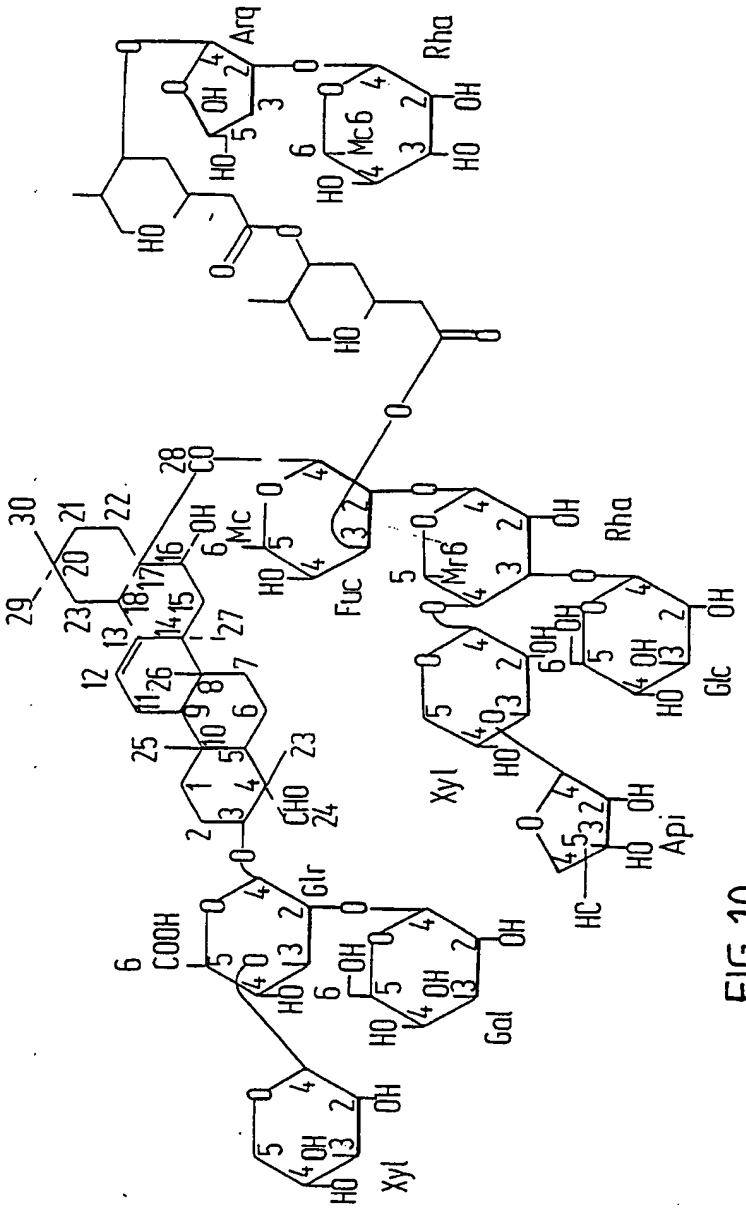
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FIG. 10

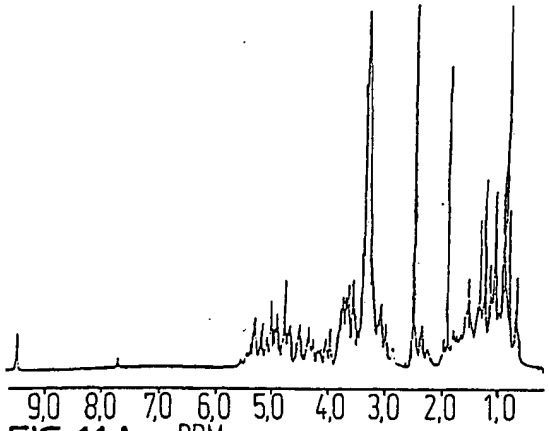

FIG.11A

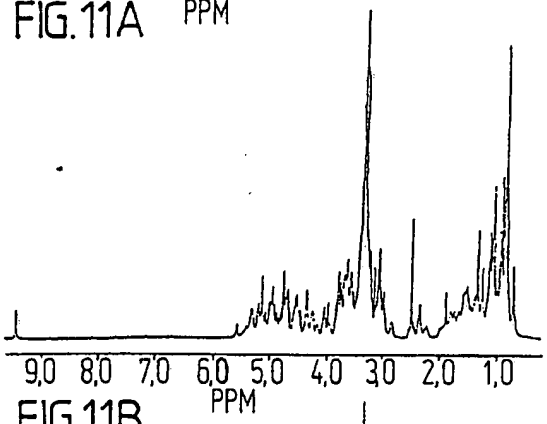

FIG.11B

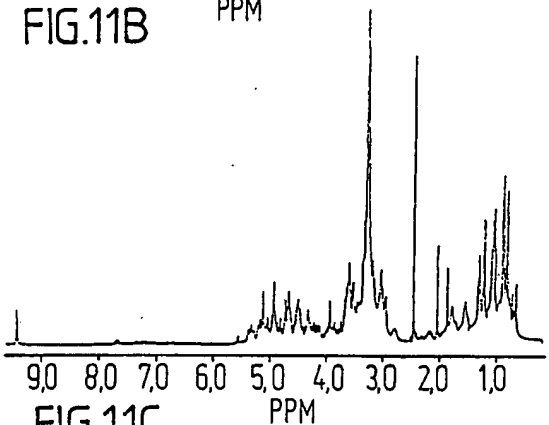

FIG.11C

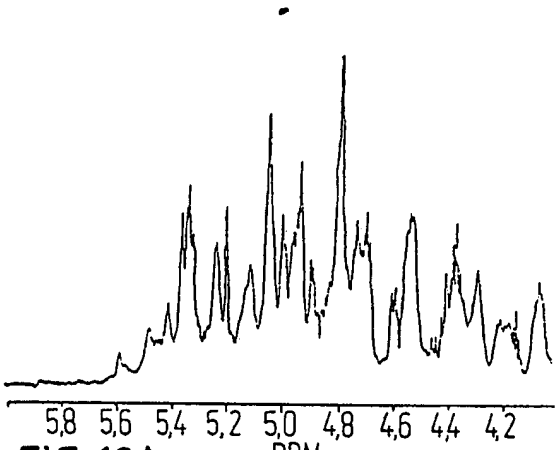

FIG. 12A

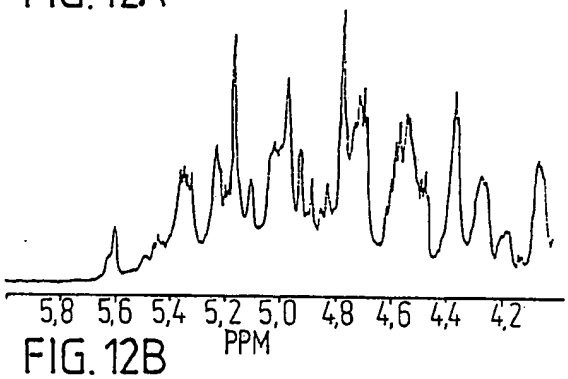

FIG. 12B

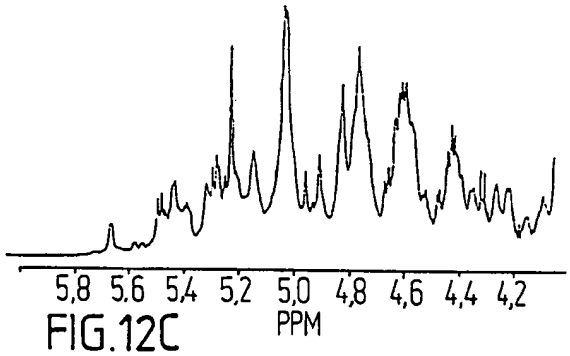

FIG. 12C

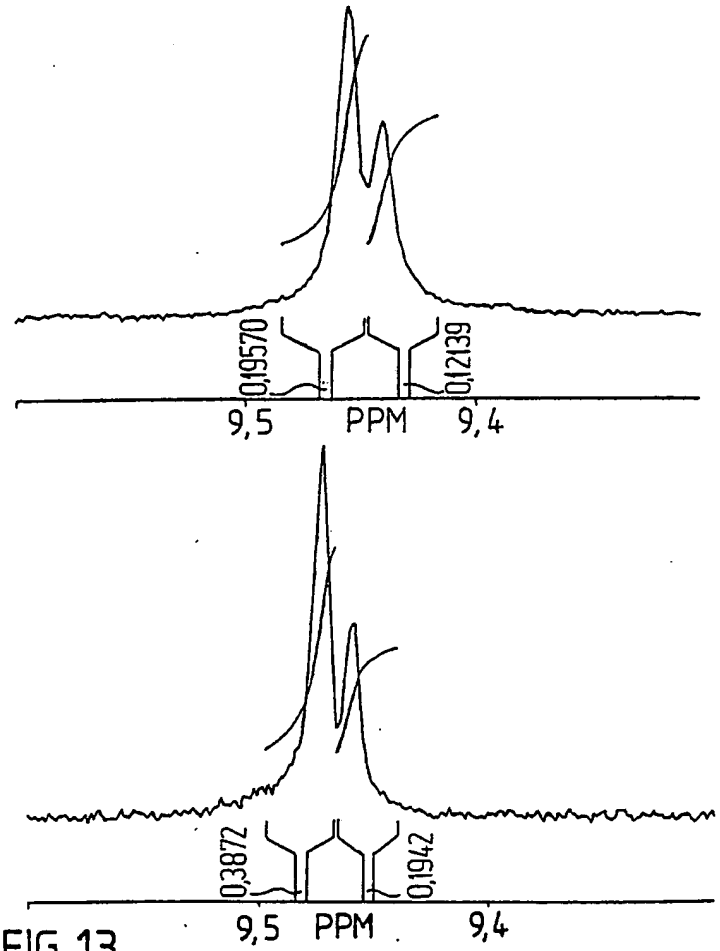
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FIG. 13

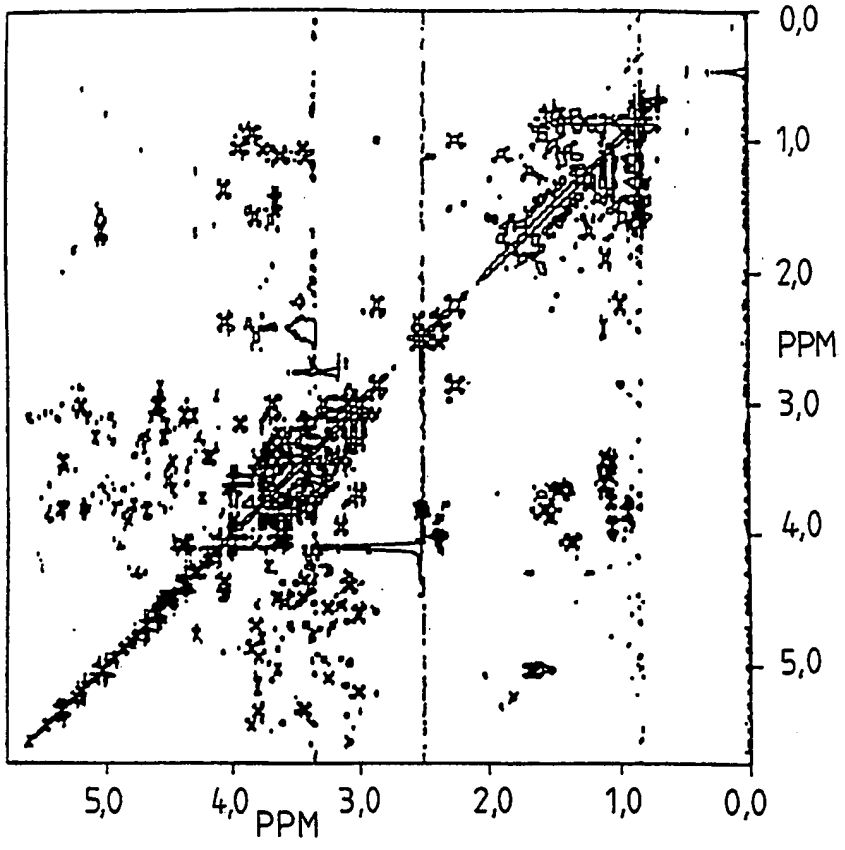
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FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 89/00528

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: A 61 K 39/39, C 07 J 17/00, 63/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC4	CA Search A 61 K; C 07 J

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Int. Archs Allergy appl. Immun., Vol. 67, 1982 R. Bomford: "Studies on the Cellular Site of Action of the Adjuvant Activity of Saponin for Sheep Erythrocytes ", see page 127 - page 131 see esp. p. 130	1
Y	--	1
Y	Biotechnology and applied Biochemistry, Vol. 10, 1988 K. Lövgren et al.: "The Requirement of Lipids for the Formation of Immunostimulating Complexes (Iscoms) ", see page 161 - page 172 see esp. p. 164-167	1
	--	
X	EP, A1, 0 231 039 (DE STAAT DER NEDERLANDEN VERTEGENWOORDIGD DOOR DE MINISTER VAN WELZIJN, VOLKSGEZONDHEID EN CULTUUR) 5 August 1987, see the whole document	7
Y	--	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th December 1989	1989 -12- 14
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	WO, A1, 88/09336 (CAMBRIDGE BIOSCIENCE CORPORATION) 1 December 1988, see esp. p. 6-8 --	11
A	US, A, 4 806 350 (JAY D. GERBER) 21 February 1989, see the whole document --	1-5
A	US, A, 4 101 652 (A. BONATI) 18 July 1978, see the whole document --	1
A	Int. Archs Allergy appl. Immun., Vol. 63, 1980 R. Bomford: "Saponin and Other Haemolysins (Vitamin A, Aliphatic Amines, Polyene Antibiotics) as Adjuvants for SRBC in the Mouse", see page 170 - page 177 --	1-5
A	Infection and Immunity, Vol. 56, No. 2, February 1988 Gideon F.A. Kersten et al.: "Incorporation of the Major Outer Membrane Protein of Neisseria gonorrhoeae in Saponin-Lipid Complexes (Iscoms): ChemicalAnalysis, Some Structural Features, and Comparison of Their Immunogenicity with Three Other Antigen Delivery Systems", see page 432 - page 438 --	1-5
A	Archiv für die gesamte Virusforschung, Vol. 44, 1974 K. Dalsgaard: "Saponin Adjuvants ", see page 243 - page 254 --	12
X	Chemical Abstracts, volume 108, no. 20, 16 May 1988, (Columbus, Ohio, US), see page 398, abstract 173547k, & JP,, 62205025 (Triterpenoid saponins as antiulcer agents.) 9 September 1987 --	11

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows: The claims 1-14 define two independent inventions, namely: Claims 1-10, 13-14 drawn to a matrix, comprising at least one lipid and at least one saponin, a process for preparing the matrix, a vaccine comprising the matrix and a kit for medicine use. Claims 11-12 drawn to specified glycosylated triterpenoid saponins and a process for preparing those.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 89/00528

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
08/11/89

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